The TRPM2 ion channel in nucleotide-gated calcium signaling

vorgelegt von
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INTRODUCTION TO CALCIUM SIGNALING

Calcium ions play an important role in almost every aspect of cell communication. Although the levels of calcium and magnesium are found at similar concentrations in living systems, only the exclusion of calcium out of the cytosol is crucial in order to allow normal physiological function. The main difference between these two alkaline metals for physiology results from calcium’s lower affinity for water. Hence, it is more subject to react with phosphates or other high-energy molecules, which are fundamental for life. Both phosphate with its negative charge and calcium with its positive charge easily interact with charged proteins resulting in conformational changes, making both ions ideal for the modulation and transduction of information. The focus here will lie on the mechanistic aspects of calcium signaling and its properties in the biological context, which is subject to highly stringent regulatory mechanisms.

Calcium as a second messenger
High concentrations (millimolar range) of calcium in the cell would result in precipitation of phosphate, making the storing and use of energy in the form of ATP impossible. Therefore mechanisms have evolved, which continuously maintain a ~10,000-fold gradient across the plasma membrane, allowing calcium to function at low concentrations (nanomolar range) as a potent messenger within the cytosol. Changes in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) probably represent the most wide-spread and most important signaling event in cellular physiology, since transient elevations of [Ca\(^{2+}\)]\(_i\) directly or indirectly control and regulate a wide spectrum of cellular responses such as e.g. muscle contraction, vesicular exocytosis, enzyme activation, gene transcription, cell proliferation, and apoptotic cell death. Cells typically maintain resting [Ca\(^{2+}\)]\(_i\) at relatively low levels around 100 nM by ATP-driven sequestration of Ca\(^{2+}\) into intracellular stores through the sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) or extrusion to the extracellular space through the plasma membrane Ca\(^{2+}\) ATPase (PMCA). Both of these compartments have ~10,000-fold higher levels of Ca\(^{2+}\) around 1 mM and this can be mobilized by the opening of Ca\(^{2+}\)-permeable ion channels, which allows Ca\(^{2+}\) to flow down its large concentration gradient. Furthermore, the gradient is stabilized through Na\(^+\)/Ca\(^{2+}\) and Na\(^+\)/Ca\(^{2+}\) +K\(^+\) exchangers in the plasma membrane.
membrane. The compartmentalization of calcium through intracellular organelles increases the resolution of this messenger, as diffusion of calcium ions is fairly low⁸.

**Information is processed through calcium-binding motifs**

For the transduction of information, calcium-chelating proteins evolved to guard and translate these signals. Hundreds of different proteins contain the EF-hand motif, the most prominent calcium-binding structure, whose Helix-turn Helix structure is represented in various functional units of proteins ranging from ion channel regulators to DNA-binding proteins⁹, ¹⁰. For example one of the main players in calcium-signaling is the highly conserved calmodulin (CaM), whose function is regulated via EF-hand motifs, encoded through multiple genes within the mammalian genome¹¹. CaM serves as an adaptor protein for the information given by calcium, subsequently acting on a highly customized recipient represented by hundreds of target proteins containing CaM-binding sites. Thus information transferred by calcium leads to stringent and specific responses through targeted proteins.

In addition to the above mechanism mediated through calmodulin, calcium can also directly act on proteins with other binding sites, for example C2 domains that lead to e.g. neutralization of charge resulting in the translocation of proteins¹². C2 domains are common in signal transduction molecules including well-established members like protein kinase C (PKC)¹³. Other calcium sensors, including calpain, a Ca²⁺-dependent cysteine protease, and calcineurin, a Ca²⁺/calmodulin-dependent protein phosphatase, are tightly regulated, as their physiological activation is crucial to a wide variety of cellular processes, such as fertilization, proliferation, development, learning, and memory¹⁴. All these calcium-binding structures play a fundamental role in signal transduction and calcium homeostasis, and are therefore strictly regulated by multiple cellular processes in living organisms¹⁵.

**Calcium signaling across the plasma membrane**

Depending on the physiological context, calcium signaling can occur directly across the plasma membrane through a variety of ion channels, which exhibit a large diversity of gating properties. Ion channels can be voltage-gated¹⁶, metabotropic-gated¹⁷, ¹⁸, store-operated¹⁹, mechano-sensitive²⁰, ligand-gated²¹ or even light-activated²². All of these are
represented by large classes of ion channels and contribute to calcium signaling in a highly differentiated manner, depending on their physiological context. In contrast to that, calcium signaling, within intracellular compartments, is rather limited to very few calcium release channels.

**Classical calcium release channels**

A ubiquitous mechanism for calcium release out of stores such as the endoplasmic reticulum (ER) is mediated through the inositol 1,4,5-trisphosphate (IP$_3$) receptor. Wide ranges of stimuli, including the interaction of G-protein- or tyrosine kinase-linked receptors cause the activation of phospholipase C (PLC). Membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP$_2$) is hydrolyzed by PLC, which generates IP$_3$ and diacylglycerol (DAG). IP$_3$ diffuses through the cytoplasm and binds to the IP$_3$ receptor, located in intracellular stores, causing calcium release. Activation of IP$_3$ receptors represents a highly dynamic process, which is strongly regulated by cytosolic calcium itself, where low concentrations of calcium facilitate the channel and high concentrations inhibit the channel$^{23}$. Furthermore the channel is gated in a rather complex way by a wide range of ligands, including ATP, which mostly modulate the channel’s sensitivity to calcium$^{24}$.

Another crucial element in the generation and modulation of intracellular calcium signals is the activity of the ryanodine-receptor (RyR), which, similar to the IP$_3$ receptor, is a dedicated calcium release channel. Ryanodine-receptors are primarily gated by calcium itself and mediate calcium induced-calcium release (CICR) from the ER and sarcoplasmic reticulum (SR). RyRs are expressed in most cell types, but have an essential role in muscle contraction$^{25}$. In excitable cells like myocytes, RyRs can cross-talk either directly or indirectly with L-type calcium channels located in the plasma membrane$^{26}$. Multiple endogenous factors, like CaM binding and cyclic ADP-ribose (cADPR) signaling can influence the activation, yet their physiological role is poorly understood$^{27}$.

**Information through calcium can be mobilized and processed from different sources**

In electrically excitable cells, which are capable of generating action potentials (AP), calcium (Ca$^{2+}$) is predominantly mobilized from the extracellular milieu. This influx is dependent on electrical activity that is orchestrated by the interplay of voltage-dependent sodium (Na$^+$), Ca$^{2+}$ and potassium (K$^+$) channels. Receptor-mediated Ca$^{2+}$ release from
intracellular stores can participate in shaping firing patterns and may also act as a localized signaling mechanism such as e.g. in dendritic spines. In some excitable cells, particularly those with a lower surface to volume ratio such as cardiac or skeletal muscle cells, the intracellular stores form an extensive network and represent the principal Ca\textsuperscript{2+} source. Here, the electrical activity acts as a trigger mechanism to cause depolarization- or calcium-induced Ca\textsuperscript{2+} release from the sarcoplasmic reticulum, which ensures a rapid and uniform increase in Ca\textsuperscript{2+} across the cytoplasm.

The relative role of Ca\textsuperscript{2+} release from intracellular stores and influx across the plasma membrane in shaping the [Ca\textsuperscript{2+}], response of a given cell type is determined by the extent and storage capacity of the endoplasmic reticulum (ER), the balance between extrusion and sequestration, and the ion channel complement of the plasma membrane. Given that the Ca\textsuperscript{2+} contents of stores is finite and some extrusion inevitably occurs, it is not surprising that Ca\textsuperscript{2+} influx is a critical component of Ca\textsuperscript{2+} signaling in practically all non-excitile cells.

**Calcium signaling in the complex network of cell specific physiology**

Ca\textsuperscript{2+} influx in non-excitile cells (i.e. cells that do not generate APs) has been studied in great detail over the past two decades and it seems clear that in most, if not all of these cells, Ca\textsuperscript{2+} release from stores and Ca\textsuperscript{2+} influx are intimately linked through a process termed capacitative or better store-operated Ca\textsuperscript{2+} (SOC) entry. Although nonexcitable cells possess multiple mechanisms for Ca\textsuperscript{2+} influx, some of which are not linked to store depletion, SOC has emerged as the most powerful and most ubiquitous mechanism for Ca\textsuperscript{2+} entry.

A specific small calcium selective current termed I\textsubscript{CRAC} (calcium release-activated current) had been detected upon depletion of stores. Just recently the molecular components of the calcium channel and store sensor have been revealed. Upon store depletion, the sensor STIM1, located in the ER moves close to the plasma membrane, where it co-associates with Orai\textsuperscript{1, 30, 31}, also named CRACM1, which is likely forming the pore of the so-called CRAC channel. Interestingly, this mechanism not only provides the function of simply refilling the stores with calcium, it also mediates long term signals like transcriptional regulation, which can be triggered by calcium signals including I\textsubscript{CRAC}. This mechanism has been described during the activation of T-
lymphocytes by dendritic cells\textsuperscript{34}. Upon contact with antigen-presenting cells (APC), the T-cell receptor (TCR) is stimulated causing IP\textsubscript{3}-mediated formation of immunological synapses (IS) and recruitment of I\textsubscript{CRAC}. Both Stim1 and Orai1 co-localize only at the area of contact between T-cell and APC. E.g. mutation in Orai1 found in human SCID (severe combined immune deficiency) patients show impaired gene regulation upon T-cell activation, demonstrating the importance of this mechanism for the calcium-mediated gene regulation\textsuperscript{29}. In this example, calcium is able to act as a slow messenger, mediating long-term regulatory signals though modulation of transcription.

Another example for the importance of calcium signaling is the insulin-secreting machinery in beta cells of the pancreas, which is subject of this thesis. There, the insulin release is regulated by calcium acting as a fast messenger\textsuperscript{35}. In order to trigger the metabolization of glucose into glycogen, glucose is taken up by glucose transporters into pancreatic beta cells\textsuperscript{36}. This uptake is backed up by phosphohexokinases, which maintain the glucose gradient by metabolizing it into glucose-6-phosphate\textsuperscript{37}. As a result of metabolization through the respiratory chain, the ratio of ADP/ATP changes, which shuts down the ATP-sensitive K\textsubscript{ATP} channel\textsuperscript{38, 39}. This causes a depolarization of the membrane, which leads to a vast calcium influx by the activation of voltage-gated calcium channels. Furthermore, this influx leads to a complex signal cascades, involving calcium-induced calcium-release (CICR), triggering both RyR and IP\textsubscript{3} receptors and resulting in calcium oscillations\textsuperscript{40}. This interplay between calcium store and plasma membrane is in addition regulated by cADPR and cAMP, through, as yet, unknown mechanisms\textsuperscript{41, 42}. All these processes contribute to the induction of the insulin-secretion machinery\textsuperscript{16}.

Therefore, it is likely, that the secretion is influenced and supported by multiple calcium-conducting proteins, as well as nucleotide receptors, including TRP channels, P2Y and adenosine receptors, all of which are found to be present in pancreatic beta cells\textsuperscript{43, 44}. Their distinct function in the complex network of glucose sensing, metabolism and insulin secretion, is to be elucidated.
**Introduction to TRP ion channels**

A family of ion channels, the so-called transient receptor potential (TRP) channels, has emerged as a potential novel player in the calcium-signaling process. The TRP family is divided into two groups and seven subfamilies. The group 1 comprises TRPC, TRPV, TRPM, TRPN and TRPA subfamilies, whereas group 2 includes TRPP and TRPML subfamilies. The number of TRP channel family members varies depending on species: 27 in man, 28 in mouse, 17 in *C. elegans* and 13 in *D. melanogaster*. The TRP superfamily of cation channels, which only recently has been fully classified, displays greater diversity in activation mechanisms and selectivity than any other ion channel family. Similar to voltage-gated potassium channels, TRP channel subunits consist of six membrane-spanning regions, forming a putative pore between segments five and six. Functional channels are likely formed by tetramers with N- and C-termini being located intracellularly, and can be associated with regulatory proteins. TRP channels share considerably little sequence homology, are often voltage-regulated and are likely to play a role in the regulation of Na\(^+\) and Ca\(^{2+}\)-concentrations in excitable as well as non-excitable cells. The activation mechanism of TRP channels is diverse, ranging from receptor activation through G-protein-mediated PLC products like diacylglycerols (DAG) and phosphoinositides. Various ligand-induced activations have been reported ranging from exogenous small molecules, including natural and synthetic compounds like icilin, capsaicin, menthol, 2-APB and endogenous activators mostly derived from nucleotides like adenosine diphosphoribose, β-NAD\(^+\) and ATP, as well as inorganic metals like calcium and magnesium. Direct activation by temperature or physical stress and coupling to receptors indicate a role of TRP channels in sensory physiology. TRP channels have been reported to play be involved in taste, touch, olfaction, hearing, thermo-regulation and hygrosensation; for example, TRPV1 has been reported to play a role in nociception, TRPM8 in temperature sensation and TRPM7 in osmotic regulation.

**TRPM2 in the network of calcium signaling**

This thesis focuses mainly on the transient melastatin-related receptor potential channel 2 (TRPM2) and its most potent agonist ADP-ribose (ADPR). TRPM2, first described in 1997, is one of the very few proteins known to combine channel function as well as
enzymatic function in one molecule. Its C-terminal domain shows sequence homology to the mitochondrial NUDT9 pyrophosphatase\textsuperscript{58}, which hydrolyses ADPR to ribose-phosphate and adenosine monophosphate and water. The gating mechanism was first revealed by enzymatic assays demonstrating the activity of the channel, though displaying reduced enzymatic activity through the NUDT9 homology region (NUDT9-h) of TRPM2\textsuperscript{21}. Like most of the TRP channels, TRPM2 is highly regulated by calcium. Either through mediation of calmodulin (CaM), as TRPM2 contains CaM-binding sites or via direct binding of calcium, which in turn, strongly up-regulates the activation of TRPM2 in combination with ADPR\textsuperscript{59}. TRPM2 channels are highly expressed in cells of the immune system such as neutrophils, monocytes and dendritic cells, as well as neuronal cells\textsuperscript{60} and pancreatic beta cells, however, its physiological function remains unclear. More and more factors are found to regulate the channel’s activity. The phosphorylation of the channel can affect its activity as well as multiple small signaling molecules derived from nucleotide-converting pathways. Regulators are O-Acetyl-ADPR, nicotinic acid adenine dinucleotide (NAADP), β-nicotinamide adenine dinucleotide (β-NAD), cADPR, poly-ADPR, some of which are known to be powerful calcium-release agonists\textsuperscript{61-65}. Most of the known pathways that lead to the activation of TRPM2 eventually metabolize ADPR or a derivate. Calcium plays a crucial role in the complex pathways of cell death, e.g. calcium signaling is required for caspase activity during apoptosis. Therefore, it was not surprising that TRPM2 was found to play a role in enhancing cell death\textsuperscript{66}, due to its calcium-conducting properties. TRPM2 can either be activated through production of ADPR, mediated by PARG/PARP (poly-ADPR glycohydrolase/poly-ADPR polymerase) in response to DNA damage or through the mitochondrial release of ADPR itself\textsuperscript{67}, which also can occur prior to apoptosis. This can be demonstrated under experimental conditions by causing oxidative stress (e.g. hydrogen peroxide/reactive oxygen species (ROS)) or cytokine signaling-mediated cell death, by e.g. tumor necrosis factor alpha (TNF-alpha). Other enzymes mediating susceptibility to cell death, like the silent information regulator 2 (sir2)\textsuperscript{68}, a protein deacetylase trigger production of O-acyl-ADPR, resulting in TRPM2 activation\textsuperscript{61}. Importantly, in experiments in which TRPM2
was knocked down, the induction of programmed cell death was suppressed, suggesting a role of TRPM2 in apoptosis.

Other enzymes, unrelated to cell-death pathways, which produce TRPM2 activators, are the ectoenzymes CD38 and CD157, both of them NADases, able to convert β-NAD into cADPR and ADPR. In addition to their ecto-cellular activities, such enzymes might possibly exert intracellular functions. Knock-out mice of CD38 display impaired calcium-dependent chemotaxis in neutrophils\textsuperscript{69}, suggesting CD38 generated metabolites may regulate this function through activation of TRPM2\textsuperscript{70}. Because CD38 and related NADases synthesize TRPM2 agonist ADPR and cADPR extracellularly, it is not clear how such metabolites may be transported to act on TRPM2 NUDT9-h domain. It has been suggested that ADPR could be internalized by CD38 itself or could be transported through nucleotide transporters\textsuperscript{71}. Just recently another ADPR metabolizing pyrophosphatase was discovered in mammalian cells, which will likely be subject to future research and open up new pathways for TRPM2 function\textsuperscript{72}. However the action of ADPR and other nucleotides is not limited to activation of TRPM2. G-Protein coupled receptors in the plasma membrane, like P2Y- and Adenosine receptors mediate signals through ADPR\textsuperscript{73}, 74. These classical pathways then can mediate both, fast signals by calcium release through the production of IP\textsubscript{3} and long-term regulation through activation of protein kinase C.

**TRP-channels and calcium release**

In addition to the commonly known function of plasma membrane-resident channels, recent research also suggests a role for TRP-channels in intracellular compartments. For example the mucolipin- and polycistin-channel (TRPML1, TRPP2) are intracellular TRP-channels\textsuperscript{75,76}. TRPML1 located in stores, but the functional relevance of this has not been demonstrated yet. TRPP2 is found in kidney epithelia. Type 2 autosomal dominant polycystic kidney disease (ADPKD) is caused by a mutation in TRPP2, which effects more than 1 in 1000 live births and is the most common monogenic cause of kidney failure in man\textsuperscript{77}. Interestingly, TRPP2 resides in the ER of kidney epithelia and is activated by calcium, which results in calcium-store depletion. In addition, TRPV1 and TRPM8 have been reported to act both as calcium influx and as calcium-release channels\textsuperscript{78,79}. TRPM8's localization in either the ER or plasma membrane has been
reported to be dependent on the expression of distinct splice variants. The dual function of these four TRP channels raises the possibility that other TRP channels, including TRPM2, may serve as intracellular release channels similar to the more classical release channels, the IP$_3$ receptor and the RyR. Analogous to RyRs and IP$_3$ receptors, many TRP channels, including TRPM2, are highly regulated by calcium, the ion they conduct. It is therefore not surprising, that TRP channels emerge in the field of calcium signaling.

**Aim of the thesis**

The aim of this work was to identify and describe calcium-signaling pathways activated by extracellular and intracellular ADPR. In order to elucidate ADPR’s function in the complex networks of calcium signaling, experiments were first conducted using HEK293 cell line, overexpressing TRPM2, and later expanded to physiologically relevant primary beta cells and immune cells. To understand the novel function of TRPM2 acting as a calcium-release channel, patch-clamp techniques were combined with simultaneous single-cell calcium imaging and other biochemical methodologies. The combination of these methods made it possible to distinguish between intracellularly- and extracellularly-initiated events in nucleotide-induced calcium signaling. In addition, ADPR signaling was studied in cells from mice deficient of TRPM2 and CD38, to further elucidate the importance of these proteins in ADPR-induced calcium signaling.
MATERIAL

Agonists and antagonists and pharmacological Inhibitors
Adenosine 5’-diphosphoribose, A-0752, Sigma
8-Br-ADPR, provided by collaboration through Prof. Dr. Santiago Partida-Sanchez
Adenosine 5’-monophosphate monohydrate from yeast, A2252, Sigma
Cyclic adenosine diphosphate-ribose, C7344, Sigma
8-Bromo-cyclic adenosine diphosphate ribose 85% HPLC, B5416-250UG Sigma
Adenosine 5’-triphosphate magnesium salt, A9187, Sigma
NAADP, Nicotinic acid adenine dinucleotide phosphate sodium salt, N5655, Sigma
Guanosine 5’-β-[thio]diphosphate trilithium salt, 51113, Sigma
Ryanodine, R-6017, Sigma
Heparin, low molecular weight, bovine intestinal mucosa, H-5027, Sigma
Ionomycin calcium salt from Streptomyces conglobatus, I0634, Sigma
Hydrogenperoxide, H1008, Sigma
8-Cyclopentyl-1,3-dipropylxanthine, C101, Sigma
CGS-15943, C199, Sigma
SCH-58261, S4568, Sigma
MRS-1754, M-6316, Sigma
U-73122, U6756, Sigma
Suramin sodium salt, 862030, Biochemika
Thapsigargin, T-7459, Molecular probes
Caffeine, O1728-500, Fisher Scientific
Leukotriene B4, 20110, Cayman Chemicals

Cell culture and media
RPMI 1640, with L-glutamine, 10-040-CV, Cellgro
RPMI-1640 Medium, 30-2001, ATCC
DMEM with glucose, L-glutamine and sodium pyruvate, 10-014-CM, Cellgro
Trypsin EDTA, T4049, Sigma
Fetal bovine serum, SH 30071.03, Hyclone
Fetal bovine serum, Heat Inactivated, USA origin, sterile-filtered, insect cell culture tested, F3018, Sigma
Fetal Bovine Serum, Regular (Heat-Inactivated), 35-011-CV, Cellgro
Penicillin/Streptomycin, 10000 U Pen/ml, 10000 µg Strep/ml, Bio Wittaker a Cambrex company
Tetracycline, 87128, Sigma
Recombinant Murine GM-CSF, 315-03, Pепrotech

**Buffers and solutions**
HBSS with calcium and magnesium and without phenol red, 21-023-CV, Cellgro
PBS, 1X, 21-040-CV, cellgro
ACK-Lysing Buffer, 06-0005 DG, GIBCO
Ethylendiaminetetraacetic acid dipotassium salt dihydrate (EDTA), 03659, Fluka, Sigma
MgCl, M1028, Sigma
D-(+)-Glucose, G6152, Sigma
NaCl, S9888, Sigma
KCl, P9333, Sigma
HEPES, H3375, Sigma
L-(+) Glutamic Acid, A125, Fisher Scientific
Cesium Chloride (White Crystalline Powder/Molecular Biology), BP210-100 Fisher Scientific
Sodium hydroxide solution, 72068, Fluka, Sigma
Potassium Hydroxide, Lc19260-1, LabChem
Cesium Hydroxide, 232041, Sigma
CaCl, 1 molar, 190464K, BDH Laboratory Supply
Hcl, A144-212, Fisher Scientific

**Enzymes**
Trypsin Inhibitor from soybean, FLUKA Biochemika 93619
Collagenase D, 11088858001, Roche
Collagenase Type XI from Clostridium histolyticum, C7657, Sigma
**Cell separation reagents and tools**

Ficoll-Paque PLUS 17-1440-03, Amersham Biosciences  
Histopaque 1077, 10771-100ML, Sigma  
NycoPrep 1.077, Axis-Shield PoC AS, Oslo, Norway, produced by Fresenius Kabi Norge AS  
Dextran 500 (T-500), 17-0320-01, Amersham Biosciences  
Cell Strainer, 10 µm Nylon, 352350, BD Flacon  
Ethrane (enflurane, USP), NDC 10019-350-60, Baxter Healthcare Cooperation  
Curad Gauze Pads, Beiersdorf Inc Wilton, CT  
MiniMacs Separator, 130-090-312, Miltenyi  
MACS Separation columns, 130-042-201, Miltenyi

**Antibodies, beads and staining reagents**

MACS CD15 MicroBeads, human, 130-046-601, Miltenyi  
MACS Anti-Ly-6G Microbeads, 130-092-332, Miltenyi Biotec  
MACS CD11b Microbeads, 130-049-601, Miltenyi Biotec  
MACS Streptavidin Microbeads, 130-048-101, Miltenyi Biotec  
Affinity Purified anti-mouse CD16/32 - blocks Fc binding, 14-0161-82, eBioscience  
Biotin anti-mouse Ly-6G (Gr-1), 13-5931-85, eBioscience  
Rosette Sep Human T-cell enrichment cocktail, 15021/61, StemCell Technologies Inc  
Polyclonal anti-TRPM2 Antibody Generation  
Rabbits were immunized with a synthetic peptide CNHKTLQKVASLFGA, located in the C-terminal portion of mouse TRPM2, conjugated to KLH and serum from three bleeds as well as preimmune serum were collected.  
Goat anti-rabbit Alexa Fluor 488 and Goat anti-mouse Alexa Fluor 594, Molecular Probes  
Rabbit anti-TRPM2 Antibody, Affinity Purified, A300-413A, Bethyl Laboratories, Inc  
Paraformaldehyde, 158127, Sigma  
Triton X-100, X100, Sigma  
anti-PDI Protein Disulfide Isomerase, Invitrogen  
4'-6'-diamine-2 phenylindole dihydrochloride (DAPI), 32670, Sigma  
mounting medium ProLong gold (Invitrogen)
Transfection
Lipofectamine transfection reagent, 18324-012, Invitrogen
Stealth RNAi negative universal control, Med 45-2001, Invitrogen
Silencer FAM Labeled Negative Control siRNA #1, AM4620, Ambion
Stealth custom primer, (RNA) - 5’ UAA GCG UUC AUG CUC UUC UGC CAG C 3’
Invitrogen

Calcium dyes and chelators
Fura-2, AM F-1221, Molecular Probes, Eugene, Oregon, USA
Fura-2 pentapotassium salt, F1200, Invitrogen Molecular Probes, Eugene, Oregon, USA
BAPTA tetra potassium salt B1204, Invitrogen
BAPTA tetra cesium salt B1212, Invitrogen

Animals
C57BL/6J wild-type mice
trpm2-/- C57BL/6J mice80
cd38-/- C57BL/6J mice81

Equipment
Inverted microscope, IX70, Olympus
Confocal microscope system, MRC 1024 ES, Biorad
EPC9, HEKA
ITC-16 computer interface, Instrutech Corp. Breantneck, NY, USA
MPCU-3, Lorenz Messgerätebau
BW SSM-125, Sony
Axiovert 200, Zeiss
Vapro Pressure Osmometer 5520, Vescor
pH meter 430, Corning
DMZ-Universal-Puller, Zeitz Instrumente GmbH
Axiovert 25, Zeiss
IM-35, Zeiss
Centrifuge 5810R, Eppendorf
Model P-87, Sutter Instrument Co.
MP-225, Sutter Instrument Co.
Oscilloscope 6080D, 60 Mhz, PeakTech
Ultrachip High res. CCD, Javelin
Model MO-103, Narishde Co. LTD
47 56 38, Zeiss
47 60 05 9901, Zeiss
Scanner Power Supply, A12 L 21
Dual Wavelength Photometer, Lorenz Messgerätebau
Micromax RF, IEC
Tubes, Capillary, Art. No 34502, size 0.8-1.10x100 mm, Klimax-51, Kimble products, USA
Semiconductor protective coating, R6101, HIPEC, Dow Corning Corporation, Midland, MI, USA
Sigmacote, SL2, Sigma
List Medical, L/M-CPZ-101, ALA scientific instruments, with Axiovert 25
Monochromator: B, Till Photonics, Germany
METHODS

Cell culture and isolation

HEK-293 cells
HEK293-TRex non-transfected (wild type) and tetracycline-inducible HEK-293 flag-TRPM2-expressing cells were cultured at 37°C with 5% CO2 in DMEM supplemented with 10% fetal bovine serum. The medium was supplemented with blasticidin (5 μg/ml; Invitrogen) and zeocin (0.4 mg/ml; Invitrogen). TRPM2 overexpression was induced by adding 1 μg/ml tetracycline to the media 16-22 hours before experiments.

INS-1 cells
The insulinoma rat cell line INS-1 was kept at 37°C with 5% CO2 in RPMI containing 10% fetal bovine serum.

Isolation of pancreatic beta cells
Pancreatic beta cells were isolated from C57BL/6 wild type or CD38 knock-out mice. Adult experimental mice (10-40 g) were anesthetized by enflurane inhalation and subsequently euthanized by cervical dislocation. An incision was made in the abdomen to expose the pancreas. The pancreatic duct was clamped at the duodenal insertion with a hemostat before inserting a cannula into the duct. The pancreas was perfused with 1.5 mg/mL collagenase then isolated, placed in a conical tube and incubated at 37°C for 20 minutes. The pancreatic tissue was rinsed three times with ice-cold RPMI 1640 medium and digested tissue was filtered through a 400 μM metal sieve to separate the pancreatic islets. The islets were further purified in Histopaque overlaid with RPMI 1640 medium by centrifuging for 20 minutes at 4°C. The islets were handpicked, then digested in 0.1% trypsin-EDTA in RPMI 1640, washed three times in RPMI 1640, plated on cover slips and incubated in RPMI 1640 supplemented with 10% FBS overnight at 37°C. The experimental protocol was performed in accordance with institutional and national regulations and was approved by the Institutional Animal Care and Use Committee (IACUC), University of Hawaii.
Isolation of human blood-derived neutrophils

Human neutrophils and T cells were obtained from whole human blood donated by volunteers with protocol approval from The Queen’s Medical Center Research & Institutional Committee. Human neutrophils were isolated using a Dextran-500 sedimentation (Amersham Bioscience 17-0320-01), followed by a Ficoll Paque Plus density centrifugation (Amersham GE, Piscataway, NJ). Cells were positively selected using Macs CD15 Microbeads (130-046-601, Miltenyi Biotec GmbH, Germany). Isolated cells were kept in a medium containing RPMI and 10% fetal bovine serum at 37 °C in an incubator. Experiments were started 1 hour after isolation. To this end, 500 µl of cells were transferred into an Eppendorf tube, diluted with 500 µl external Na⁺-Ringer, centrifuged and resuspended in 500 µl Na⁺-Ringer.

Isolation of human T-lymphocytes

Human T cells were isolated using the RosetteSep™ protocol according to manufacturer’s instructions (StemCell Technologies Inc., Vancouver, Canada). Cells isolated this way were kept in standard RPMI tissue culture medium supplemented with 10% FBS at 37 °C until used for patch-clamp experiments.

Isolation of blood-derived monocytes

Adult experimental mice (10g -40g) were anesthetized by enflurane inhalation and subsequently euthanized by cervical dislocation. Periphal blood was immediately collected by cardiac puncture and pooled together with PBS Buffer containing 3 mM K-EDTA. Mononuclear cells were isolated by density centrifugation using nycoprep 1.077A. The blood suspension was carefully layered on half the volume of density media and centrifuged at 600 g for 20 minutes. Mononuclear cells were harvested from the interphase using an Eppendorf pipette. Cells were washed with PBS containing 0.5% FBS and 2 mM EDTA. Monocytes were positively selected through magnetic separation using Miltenyi Biotec MACS CD11b Microbeads. Cells were kept in RPMI 1640 containing 10% FBS, 0.2 M HEPES and Penicillin Streptomycin at 37 °C with 5% CO₂. All animal procedures were performed in accordance to federal, state, local and university guidelines, approved by the University of Hawaii’s Institutional Animal Care and Use Committee (IACUC).
Isolation of murine spleen-derived neutrophils
Spleen was removed and perfused with Collagenase D (0.15U/ml in 1x HBSS) by syringe injection, chopped into small pieces by sharp scalpels and incubated at 37° C for 30 minutes. Debris and cell clusters were removed by filtration through layers of gauze pads. Cells were washed with PBS x 1 containing 0.5 % FBS and 2 mM EDTA, resuspended and positive selected through Miltenyi magnetic separation using combination of Anti-Ly-6G-Biotin MicroBeads and Anti-Biotin MicroBeads following manufacturers instructions. Cells were kept in RPMI containing 26 mM NaHCO3, 200 Units of Penicillin per liter and 200 µgr of Streptomycin per liter and 0.02 molar of HEPES at 4.7e⁻⁵/ml.

Neutrophil isolation from bone marrow (mouse)
Bone Marrow Neutrophils were purified from femoral and tibial bone marrow. Bones were collected, broken opposite of bending and cells were flushed out with 5 to 10 ml FACS buffer (PBS+2% Bovine serum or BSA) using 23 G needle. Cells were washed and clumps were broken by repeated aspiration using 18G needle and 20 ml syringe. Cells suspension was further positively selected with biotynyled anti-Gr-1 antibody and MACS Streptavidin Microbeads using Miltenyi magnetic cell separation according to manufacturers protocol. Cells were kept in RPMI containing 10 % FBS, 26 mM NaHCO3, 200 Units of Penicillin per liter and 200 µgr of Streptomycin per liter and 0.02 molar of HEPES at 4.7e⁻⁵/ml.

Culture of bone marrow-derived dendritic cells
Bone marrow-derived cells were obtained as described for the bone marrow-derived neutrophils. Cells were treated using ACK lysing buffer. Cells were kept in RPMI high glucose containing 7% FBS at 3x10⁵ cell/ml at a total of 20ml of cellular suspension in 150 mm X 25mm plates. To differentiate precursors to immature DCs, mouse bone marrow cells supplemented with 20 ng/ml GM-CSF for 5 days
**INS-1 siRNA experiments**

Three siRNA 25-mers (20 nM) matching the rat TRPM2 sequences were obtained from Invitrogen using their BLOCK-iT™ RNAi Designer for custom Stealth siRNA duplex oligoribonucleotides and diluted in DPEC treated water (1 ml) to a concentration of 20 pmol/µl. Uptake of RNA was confirmed using AM4620 Silencer® FAM™ labeled Negative Control #1 siRNA from Ambion (20 pmol/µl) with Olympus IX70 Inverted Microscope fluorescence microscopy. Stealth RNAi Negative Control Medium GC Duplex (Cat. No. 12935-300, Invitrogen) (20 pmol/µl) was used as a control for sequence-independent effects. INS-1 cells were seeded in six well tissue culture plates at 2x10⁵ cells per well in 2 ml RPMI containing 10% FBS. Cells were cultured in a CO₂ incubator at 37°C until 70-80% confluence. Cells were transfected according to manufacturers protocol using Invitrogen Lipofectamine 2000. Cells were cultured after transfection for 18-24 hours, trypsinized, washed and plated on glass coverslips. Patch clamp and imaging experiments were performed 40-60 hours post-transfection. Screened sequence of efficient knock-down of rat-TRPM2 was (RNA) - 5’ UAA GCG UUC AUG CUC UUC UGC CAG C 3’.

**TRPM2 and ER fluorescence labeling in INS-1 cells.**

INS-1 cells were grown on coverslips. The cells were washed with PBS, fixed with 2% paraformaldehyde (sigma) and permeabilized with 0.2% Triton X-100 (0.05%) for 5 min at room temperature. Slides were blocked with 10% of goat serum and then incubated with anti-mTRPM2 specific serum or preimmune serum (1:500) and anti-PDI (Invitrogen 1:1000), mouse antibody directed against the ER-associated protein disulfide isomerase, for 2 hours at 37°C. Goat anti-rabbit Alexa Fluor 488 and Goat anti-mouse Alexa Fluor 594 (Molecular Probes 1:1000) were used as a secondary antibodies. Nucleic acids were stained with 4’-6’-diamine-2 phenylindole dihydrochloride (DAPI)-containing mounting medium ProLong gold (Invitrogen) Slides were mounted and cells visualized with a Zeiss ApoTome Axiovert 200 imaging microscope at 63x using an Axiocam MRM CCD camera and the Zeiss AxioVision software.
**TRPM2 immunofluorescence in mouse neutrophils and dendritic cells**

For TRPM2 staining, bone marrow-derived dendritic cells and neutrophils were attached on coverslips that were pre-treated with poly-L-lysine and fixed with 2% paraformaldehyde for 15 min at RT. Cells were permeabilized with 0.2% Triton X-100 for 5 min. Samples were rinsed with PBS 1x and blocked with 10% goat serum or 2% BSA for 30 min at 37 C. Cells were incubated polyclonal rabbit anti-human TRPM2 antibody, for 2 hours at 37 C in dark an in a humid chamber. The Alexa flour 488 anti-rabbit IgG was used as secondary antibody. Samples were maintained using the Prolong Gold antifade reagent with DAPI (Invitrogen). Samples were analyzed using the Zeiss 510 LSM META Confocal Laser Scanning Microscope and Zeiss LSM Image Browser program.

**Electrophysiology and fluorescence measurements**

**Voltage clamp protocols**

Patch-clamp experiments were performed in the whole-cell configuration at 21-25 °C. Patch pipettes were pulled from Kimax glass capillaries (Kimble Products, Fisher Scientific, USA) on a DMZ-Universal Puller (DAGAN, Minneapolis, MN), and had resistances of 2-5 MΩ. Data were acquired with Pulse and PatchMaster software controlling an EPC-9 amplifier. Voltage ramps of 50 ms spanning the voltage range of –100 to +100 mV were delivered at a rate of 0.5 Hz, typically over a period of 100 s. For Mg^{2+}/ATP-regulated TRPM7-like current cells were perfused with regular Cs-based solution supplemented with 10 mM Cs-BAPTA. In the measurements of $I_{\text{CRAC}}$ ramps were run from –150 mV to +150, data points obtained at –80 mV representative for inward currents. The holding potential was 0 mV. Voltages were corrected for a liquid junction potential of 10 mV. Currents were filtered at 2.9 kHz and digitized at 100 µs intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp. The low-resolution temporal development of currents for a given potential was extracted from individual ramp current records by measuring the current amplitudes at voltages of –80 mV. Data were analyzed using PulseFit or FitMaster (HEKA, Lambrecht, Germany), and IgorPro (WaveMetrics, Lake Oswego, Or). Data were exported from PulseFit or FitMaster without leak subtraction with the exception of pancreatic beta cell dose/response measurements and $I_{\text{CRAC}}$ measurements in mouse
monocytes where the first data point was subtracted from obtained currents. Currents were normalized to cell size in pF. Basal currents were taken from the averaged and normalized current plateau phase at a compound concentration that did not activate TRPM2 currents (100 nM ADPR, 300 nM cADPR, 0 Ca$^{2+}$). Background currents ranged between −5 and −15 pA/pF at −80 mV. The average cell size of human neutrophils was 2.3 ± 0.08 pF (n = 40), of human T lymphocytes 1.7 ± 0.05 pF (n = 75), mouse monocytes around 4-8 pF, primary beta cells 6-12 pF, INS-1 cells 8-14 pF, dendritic cells 10-30 pF and HEK-293 15-25 pF. Where applicable, statistical errors of averaged data are given as means ± S.E.M. with n determinations. Single ramps were plotted as current-voltage relationships (IVs) and were not leak-subtracted. External solution changes were performed using a wide-mouthed glass pipette controlled by a pneumatic pressure devise (Lorenz Messgerätebau, Katlenburg-Lindau, Germany).

**Fluorescence measurements**

Fluorescence signals were sampled at a rate of 5 Hz with a photomultiplier-based system using a monochromatic light-source (TILL Photonics, Gräfelfing, Germany). Emission was detected with a photomultiplier whose analog signals were sampled by a digital-analog interface (ITC-16, Instrutech, New York) and processed by X-Chart software (HEKA, Lambrecht, Germany). Fluorescence ratios were calculated into free intracellular Ca$^{2+}$ concentration based on calibration parameters derived from patch-clamp experiments with calibrated Ca$^{2+}$ concentrations. Three different kinds of fluorescence experiments were performed. In experiments combining patch-clamp and fluorescence experiments, cells were perfused with standard intracellular pipette solution containing 200 μM Fura-2. Balanced-Fura-2 experiments were performed by pre-loading cells with Fura-2-AM at 5 μM and for 30 minutes. In the subsequent whole-cell patch clamp experiments 200 μM Fura-2 had been added to the standard internal solution to assure continuous Fura-2 signals. In pancreatic beta cells cells were held in the whole cell configuration at −70 mV to prevent voltage gated calcium channel activation upon extracellular-induced calcium-release stimulus. For intact-cell Ca$^{2+}$ measurements, cells were loaded with 5 μM Fura-2-AM for 30 minutes.
**Fura-2 Ca\(^{2+}\) measurements and perforated patch**

For Ca\(^{2+}\) measurements, cells were loaded with 5 µM Fura-2-AM (acetoxymethylester, Molecular Probes) for 30 min in media at 37 °C. Fura-2 experiments were performed using Fura-2-AM pre-loaded cells and parallel perforated-patch clamp experiments, where 300 µM amphotericin B (Sigma, freshly prepared from 30 mM stock in DMSO) had been added to the standard internal solution. To this end, cells were kept in the cell-attached mode at a holding potential of 0 mV until the series resistance was less than 20 MΩ (within 10 min.), then the standard ramp protocol (see above) was started.

The cytosolic calcium concentration of individual patch-clamped cells was monitored at a rate of 5 Hz with a dual excitation fluorometric system using a Zeiss Axiovert 200 fluorescence microscope equipped with a 40x LD AchromPlan objective. The monochromatic light source (monochromator B, TILL-Photonics) was tuned to excite Fura-2 fluorescence at 360 and 390 nm for 20 ms each. Emission was detected at 450-550 nm with a photomultiplier, whose analog signals were sampled and processed by X-Chart software (HEKA, Lambrecht, Germany). Fluorescence ratios \(F_{360}/F_{390}\) were translated into free intracellular calcium concentration based on calibration parameters derived from patch-clamp experiments with calibrated Ca\(^{2+}\) concentrations.

**Single channel measurements**

Single channel recordings were performed in the whole-cell configuration using standard external solutions. For single channel acquisition, K\(^+\) ions were replaced with cesium (Cs\(^+\)). A threshold concentration of 100 nM or 200 nM ADPR in unbuffered intracellular conditions was used to evoke a low level of channel activity over time. Ramps from –100 mV to 100 mV over 20 s were applied continuously, recorded at a gain of 50 mV/pA and filtered at 50 Hz. Due to the slight outward rectification visible in the measurements, linear fits to the data were performed from either –100 mV to 0 mV or from 0 mV to +100 mV to evaluate the single channel conductance in pS.
Solutions

For patch-clamp experiments, cells were kept in standard external solution (in mM): 140 NaCl, 2.8 KCl, with 1 CaCl$_2$ or without CaCl$_2$, 2 MgCl$_2$, 11 glucose, 10 HEPES·NaOH (pH 7.2 adjusted with NaOH/CsOH, 300-320 mOsm), supplemented with pharmacological inhibitors, suramin, CGS-15943, U73122, Thapsigargin, DPCPX, SCH-58261 or MRS-1754. In some experiments external solution containing β-NAD, ATP, 8-Br-ADPR, caffeine, ADPR, cADPR, charbachol, thrombin, LTB4 or ionomycin was applied. Standard pipette-filling solutions contained (in mM): either 120- 140 K-glutamate or 120- 140 Cs-glutamate, 8 NaCl, 1 MgCl$_2$, 10 HEPES·KOH (pH 7.2 adjusted with KOH, 290-310 mOsm). ADPR, cADPR, NAADP, H$_2$O$_2$, sodium heparin, Ryanodine, GDP-β-S, Capsaicpine, AMP-monophosphate-monohydrate, 8-Br-ADPR, 8-Br-cADPR or a combination thereof was added to the standard internal solution. [Ca$^{2+}$]$_i$ was buffered to 0, 100, 200, 300, 500 or 1000 nM with 10 mM BAPTA and 0, 3.1, 4.7, 5.7, 6.9 or 8.2 mM CaCl$_2$, respectively, calculated with WebMaxC (http://www.stanford.edu/~cpatton/webmaxcS.htm) or left unbuffered (no Ca$^{2+}$ buffer present). All chemicals except BAPTA (Invitrogen-Molecular Probes, Carlsbad, CA) were purchased from Sigma-Aldrich, USA.
RESULTS

Overview
In order to understand the calcium-mobilizing action of ADP-ribose by either acting on calcium influx pathways through TRPM2 or by recruiting calcium from internal stores, experiments were first carried out using an overexpression system. Therefore, HEK293 cells exogenously expressing the TRPM2 channel upon tetracycline induction were used. It was found that external ADP-ribose mediates another function besides plasma membrane currents. ADPR was also able to activate G-protein-mediated IP₃-producing pathways via P2Y receptors. Furthermore, it was demonstrated for the first time that intracellular ADPR alone was able to cause calcium release in cells overexpressing TRPM2 but not in wild-type cells. Consequently, experiments were carried out using a cell line of pancreatic origin, which endogenously expresses TRPM2. Thereby, it was demonstrated that TRPM2 was able to act as a plasma membrane channel and as a functional calcium release channel. This finding was later confirmed by testing primary mouse beta cells, where endogenous TRPM2 channels also function as both plasma membrane and release channel. Having demonstrated that TRPM2 can function as such, investigations were carried out in hematopoietically-derived cells, which have been found to express high levels of TRPM2 channel. It was found that in primary cultured dendritic cells, TRPM2 mainly functioned as a release channel. Interestingly, it was shown that in blood-derived primary human neutrophils, TRPM2 was limited to function as a plasma membrane channel. Here, a detailed electro physiological characterization was carried out. Similar properties were observed in human and mouse-derived neutrophils. Furthermore, neutrophils isolated from mice deficient in CD38 enzyme activity showed no modification of channel activity. Further investigations using mouse blood-derived monocytes showed the presence of TRPM2 currents, that were absent in a TRPM2 knock-out model. The knock-out itself did not affect other calcium influx channels such as CRAC or TRPM7.
In addition it was found that the TRPM2 antagonist adenosine-mono-phosphate had a major impact on inhibiting G-protein-mediated pathways targeting intracellular calcium stores. A screening of different agonists of calcium release pathways in multiple cell systems showed that AMP acted in a ubiquitous manner by inhibiting G-protein-mediated
pathways. This mechanism was demonstrated to be distinctly different from simply targeting the IP3 receptor. The mechanism itself remains to be elucidated.

**ADP-Ribose is a multimodal agonist for purinergic receptors and TRPM2 channels in the plasma membrane and intracellular stores of beta cells**

In order to conduct experiments aimed at resolving the intracellular action of ADPR, it first had to be deciphered which pathways ADPR would trigger from the outside. It had been reported that extracellular ADPR was able to elevate calcium levels in rat beta cell lines independent of channel-mediated calcium entry74. Another publication indicated a possible action of ADP-ribose on P2Y receptors in a different cell model, though this process had not been linked to calcium store depletion73.

**Extracellular ADPR triggers calcium release through P2Y receptors in HEK293 cells**

To assess the effects of extracellular ADPR, intact HEK293 cells overexpressing TRPM2 (see methods) were investigated. Cells were loaded with Fura-2-AM and ADPR was applied, while recording [Ca$^{2+}$]$_i$. ADPR application consistently produced a transient [Ca$^{2+}$]$_i$ signal in both wild-type (Fig. 1B) and TRPM2-expressing cells (Fig. 1A). This response was concentration-dependent with a threshold of ~100 µM ADPR and neither required the presence of extracellular Ca$^{2+}$ (Fig. 1A) nor TRPM2 expression (Fig. 1B), demonstrating that it originated from release of Ca$^{2+}$ from intracellular stores through a pre-existing signaling pathway that is independent of TRPM2. However, cells overexpressing TRPM2 seemed to produce slightly faster and larger responses to extracellular ADPR, possibly indicating enhanced Ca$^{2+}$ release activity. The lack of a more pronounced and sustained Ca$^{2+}$ signal in these cells, even when Ca$^{2+}$ was present in the medium (Fig. 1A), suggests that TRPM2 channels in the plasma membrane were not activated to a significant extent when ADPR was applied extracellularly.

Nucleosides and different ribosylated nucleotide derivatives have previously been shown to activate calcium signaling pathways related to purinergic receptors74. In HEK293 cells, ATP can cause production of inositol 1,4,5 trisphosphate (IP3) and a rise in [Ca$^{2+}$]$_i$ that is inhibited by P2Y receptor antagonists82-84. Therefore the effects of suramin, a non-selective P2Y receptor antagonist, on ADPR-induced Ca$^{2+}$ signals in Fura-2-loaded TRPM2-expressing cells were examined. Cells were preincubated for 15 min in 100 µM suramin. The response of elevated calcium was completely suppressed at a concentration
of even 1 mM ADPR (Fig. 1A), suggesting that the external ADPR-induced signal was mediated through members of the P2Y receptor family.

Indeed, when HEK293 cells were first exposed to another P2Y receptor agonist, ATP (100 µM), and then to ADPR, the ATP-induced Ca^{2+} release leads to emptied Ca^{2+}, so that subsequent application of ADPR failed to elicit a [Ca^{2+}]_{i} response (Fig. 1C). Together, these results indicated that ADPR might act through the PLC signaling pathway. This was further tested by systematical and sequential inhibition of the G-protein/PLC/IP_{3} signal transduction pathway in patch-clamp experiments (Fig. 1D). Interfering with G-protein coupling by internal perfusion of HEK293 cells with 500 µM GDP-β-S for 100 seconds completely blocked ADPR-induced Ca^{2+} release. Likewise, pre-incubation of cells with the PLC inhibitor U73122 (10 µM) also eliminated receptor-mediated Ca^{2+} release, induced by superfusing cells with ADPR. Lastly, the IP_{3} receptor was directly blocked by perfusing cells internally with 100 µg/ml heparin. This also prevented ADPR-induced Ca^{2+} release. These data demonstrated that ADPR could act as a first messenger through G-protein-coupled receptors that activate the PLC pathway.

**Effects of extracellular NAD^{+} and cADPR in HEK293 cells**

In further experiments, the specificity of the ADPR effect was assessed. ADPR can be produced extracellularly from its precursors NAD^{+} or cADPR through the action of the ectoenzyme CD38^{85}. Even at high millimolar concentrations, neither NAD^{+} nor cADPR were effective in producing Ca^{2+} signals (Fig. 1B), suggesting that these molecules are not effective agonists for P2Y receptors and that HEK293 cells may not express sufficiently high levels of CD38 to produce significant amounts of ADPR from these metabolites. Even at the highest concentration of NAD^{+} (10 mM) only 1 out of 6 cells responded with a small Ca^{2+} transient, suggesting that this was the threshold concentration at which Ca^{2+} release might begin to occur. Since previously it was reported that the batch of NAD^{+} used in this study contains ~3% contamination of ADPR^{63}, it was surmised that the Ca^{2+} release observed at 10 mM NAD^{+} might have been caused by the ~90 µM contamination of ADPR. Likewise, only 2 out of 8 cells responded with Ca^{2+} release at 10 mM cADPR (Fig. 1B), suggesting that this represents its threshold concentration.
Although another report has claimed that cADPR might contain up to 25% contaminating ADPR\textsuperscript{86}, these functional results would suggest that, if the $\text{Ca}^{2+}$ response at 10 mM cADPR were caused by contaminating ADPR, the contamination in this cADPR preparation would be equivalent to $\sim$100 µM ADPR, i.e. $\sim$1% at most. Taken together, these data suggest that the $[\text{Ca}^{2+}]_i$ signals evoked by extracellular ADPR are specific and mediated by P2Y receptors coupling to the G-protein/PLC/IP\textsubscript{3} pathway, making ADPR a genuine first messenger. However, this would not rule out the possibility that intracellular ADPR may act as an intracellular second messenger as well and generate $\text{Ca}^{2+}$ release via TRPM2 or otherwise.

**Effect of intracellular ADPR in HEK293 wild-type and TRPM2-expressing cells**

The next experiments investigated a possible additional role of ADPR as an intracellular second messenger for $\text{Ca}^{2+}$ release. Being aware of the above-described receptor-mediated effects of ADPR and its ability to activate TRPM2 $\text{Ca}^{2+}$ influx channels through internal perfusion\textsuperscript{21}, experiments were designed in which $\text{Ca}^{2+}$ release through the upstream P2Y pathway and $\text{Ca}^{2+}$ influx through TRPM2 channels in the plasma membrane were eliminated by the presence of suramin and the absence of $\text{Ca}^{2+}$ in the extracellular medium, respectively. Cells were loaded with Fura-2-AM and then patch-clamped in the whole-cell configuration to introduce 0.1 to 1 mM ADPR intracellularly. Patch pipettes additionally contained 200 µM Fura-2 to replenish the pre-loaded Fura-2 and maintain the ability to measure $[\text{Ca}^{2+}]_i$ during whole-cell recordings. As soon as cells were perfused with 100 µM or 1 mM ADPR, TRPM2-expressing cells responded with clear $\text{Ca}^{2+}$ release signals (Fig. 1E), whereas ADPR was ineffective in wild-type cells (Fig. 1F), suggesting that ADPR-induced $\text{Ca}^{2+}$ release via TRPM2 channels located in intracellular stores.
Figure 1: ADPR functions as purinergic receptor agonist and TRPM2 is a novel calcium release channel when over-expressed in HEK293 cells. (A) The graph shows averaged Ca\(^{2+}\) signals measured in intact HEK293 cells overexpressing TRPM2 channels (TRPM2 HEK293) in response to application of extracellular ADPR in the presence (1 mM, black trace, n = 8) or absence of extracellular Ca\(^{2+}\) (blue trace, n = 7) in the standard external solution. The concentration of ADPR in the presence of Ca\(^{2+}\) was 1 mM and ADPR was 100 μM in the absence of Ca\(^{2+}\). The red trace represents the averaged Ca\(^{2+}\) signal measured in response to application of 100 μM ADPR in the absence of extracellular Ca\(^{2+}\) and addition of 100 μM suramin (n = 6). Application started as indicated by the arrow and was maintained throughout the experimental time displayed. Cells were loaded with 5 μM Fura-2 AM at 37°C for 30 min. (B) The panel displays averaged Ca\(^{2+}\) signals in intact wild-type HEK293 cells in response to application of 1 mM ADPR (black trace, n = 7), 10 mM cADPR (red trace, n = 8) or 10 mM NAD\(^{+}\) (blue trace, n = 6) in the absence of extracellular Ca\(^{2+}\). Application and Fura-2 AM loading as described in Panel A. (C) Averaged Ca\(^{2+}\) signal measured in intact Fura-2 AM loaded TRPM2 HEK293 cells in response to application of 100 μM ATP (black bar) followed by application of 100 μM ADPR (red bar) in the absence of extracellular Ca\(^{2+}\) (n = 6). (D) The graph depicts balanced Fura-2 experiments. Averaged Ca\(^{2+}\) signal in whole cell patch-clamped TRPM2 HEK293 cells preloaded with Fura-2 AM. Whole-cell break-in was before application start (not shown). Application start of 100 μM ADPR in the absence of extracellular Ca\(^{2+}\) as indicated by the arrow. The internal solution contained 200 μM Fura-2 and additionally either 100 μg/ml heparin (blue trace, n = 6) or 500 μM GDP-β-S (green trace, n = 5). The red trace represents data where the cells were perfused with internal solution supplemented with 200 μM Fura-2 and 10 μM U73122 in the bath (n = 5). (E) The graph shows balanced Fura-2 experiments. Averaged Ca\(^{2+}\) signal in whole cell patch-clamped TRPM2 HEK293 cells preloaded with Fura-2 AM. Whole-cell break-in was at the time indicated by the red arrow. Cells were kept in 0 Ca external solution and perfused with internal solution containing either 1 mM ADPR (black trace, n = 6), 100 μM ADPR (blue trace, n = 5) or 100 μM ADPR and 1 mM AMP (red trace, n = 8). 200 μM Fura-2 had been added to the internal solution in all three experimental conditions. (F) The graph shows balanced Fura-2 experiments in wild-type HEK293 cells preloaded with Fura-2 AM. Whole-cell break-in was achieved at the time indicated by the red arrow. Internal solution was supplemented with 1 mM ADPR and 200 μM Fura-2 (n = 6).

The above experiments firmly established that TRPM2, in principle, could function as an intracellular Ca\(^{2+}\) release channel in a heterologous overexpression system, prompting the question whether this function is relevant under physiological circumstances in cells that express TRPM2 natively.
**INS-1 cells as a model for endogenous TRPM2**

It had been previously reported that rat RINm5F and CRI-G1 cell lines\(^8\) natively express TRPM2. The investigations were further extended to the rat pancreatic beta cell line INS-1 as a cell model known for calcium being important in physiological function of insulin release. It was confirmed that functional TRPM2 channels were indeed expressed in the plasma membrane. Cells were perfused intracellularly with various concentrations of ADPR under ionic conditions that were as close as possible to physiological conditions, while still suppressing interference of other endogenous channels (see Methods). Perfusion of ADPR caused a rapid activation (Fig. 2A, open circles) of a linear current exhibiting the typical characteristics of TRPM2 (Fig. 2B). The ADPR-mediated currents were activated in a concentration-dependent manner with a half-maximal effective concentration (EC\(_{50}\)) of \(\sim 100\ \mu M\) ADPR (Fig. 2C) and were completely suppressed by the addition of 1 mM AMP (Fig. 2A, closed circles; 2B red trace). These data confirm that TRPM2 is functionally expressed in INS-1 cells and acts as a plasma membrane ion channel.

**Extracellular ADPR triggers calcium release through P2Y and adenosine receptors in INS-1 cells**

A similar strategy as that employed in HEK293 cells to assess the Ca\(^{2+}\) signaling mechanisms of ADPR was used in INS-1 cells. As in HEK293 cells, extracellular application of ADPR in Ca\(^{2+}\)-free solution induced Ca\(^{2+}\) release responses in INS-1 cells at a threshold concentration as low as 1 \(\mu M\) (Fig. 2D). Thus, ADPR was about two orders of magnitude more potent in activating Ca\(^{2+}\) release from INS-1 cells than in HEK293 cells. Possible reasons for the enhanced sensitivity include species differences in P2Y sensitivity, different complements of P2Y receptor subtypes, and/or the presence of additional non-P2Y receptors that are ADPR sensitive.

The possibilities were examined by antagonizing P2Y receptors with suramin and stimulating cells with 100 \(\mu M\) ADPR. While blocking P2Y receptors with suramin reduced the ADPR-induced \([Ca^{2+}]_i\) signal, it failed to completely suppress the response, indicating the presence of another cell surface receptor responsive to ADPR in this cell system (Fig. 2E). Beta cells, in addition to expressing P2Y receptors, also have been
found to express A-1 adenosine receptors$^{88-90}$, raising the possibility that they might be activated by ADPR and account for the residual Ca$^{2+}$ release activity in the presence of the P2Y antagonist suramin. This hypothesis was confirmed by experiments in which the broad adenosine receptor antagonist CGS-15943 was used. Although this compound, when applied alone, did not abolish Ca$^{2+}$ release, it caused a similar reduction of the [Ca$^{2+}$]$_i$ signal as that seen with suramin alone (Fig. 2E). However, incubating cells with CGS-15943 in combination with suramin to block both P2Y and adenosine receptors, completely abolished the ADPR-mediated Ca$^{2+}$ release signal (Fig. 2E). Since P2Y and adenosine receptors can couple to the classical receptor/G protein/PLC/IP$_3$ pathway$^{91,92}$, it was surmised that the ADPR-mediated responses in INS-1 cells were likely mediated, at least in part, by IP$_3$-induced Ca$^{2+}$ release.

Next it was examined whether the enhanced sensitivity of INS-1 cells to ADPR was caused by adenosine or P2Y receptors. To this end, cells were stimulated with a low concentration of 10 μM ADPR and adenosine receptors were inhibited with CGS-15943 or P2Y receptors with suramin. Figure 2F demonstrates that suramin was considerably more effective than CGS-15943 in suppressing the response to the low concentration of ADPR, suggesting that P2Y receptors are primarily responsible for the higher sensitivity of INS-1 cells. Since the ADPR response in HEK293 cells is also mediated through P2Y receptors, it would appear that either species differences or the P2Y receptor subtypes complements of rat INS-1 vs. human HEK293 cells account for the differences in ADPR sensitivity. HEK293 cells primarily express P2Y subtypes 1, 2, and 4$^{82}$, although a slightly differing P2Y receptor complement has been reported for these cells as well$^{93}$. INS-1 cells express subtypes 1, 2, 4, 6, and 12, which are expressed at similar levels$^{82,94}$. Thus it would seem that a specific P2Y receptor subtype complement might determine the high-affinity response to ADPR in INS-1 cells, although it was not ruled out entirely that species differences or clonal variation might play a role as well. A more extensive pharmacological profiling of P2Y receptors in INS-1 cells would be able to resolve this question.

Effects of extracellular NAD$^+$ and cADPR in INS-1 cells

In addition, the ADPR metabolites, NAD$^+$ and cADPR were tested for efficacy in evoking Ca$^{2+}$ release responses in INS-1 cells. In marked contrast to HEK293 cells,
where these molecules failed to induce Ca\(^{2+}\) release (see Fig. 1B), both NAD\(^{+}\) and cADPR were able to trigger Ca\(^{2+}\) release transients in INS-1 cells, although cADPR did so more efficiently than NAD\(^{+}\). The threshold concentration for cADPR was \(~10\) µM (Fig. 2G) and for NAD\(^{+}\) \(~30\) µM (Fig. 2I). Since these threshold concentrations were just 10 to 30-fold higher than that of ADPR, and maximal levels of contamination of these compounds with ADPR were determined being \(~1\)-3\%, the NAD\(^{+}\)- and cADPR-mediated Ca\(^{2+}\) release activity was clearly not caused by nucleotide contamination. This response was either due to a genuine agonistic action of these compounds on cell surface receptors or caused by exogenous metabolic conversion to ADPR through the NADase CD38, which is expressed at high levels in beta cells\(^{95}\), but presumably less so in HEK293 cells. One may therefore hypothesize, that the NAD\(^{+}\)- and cADPR-mediated responses are likely caused by conversion of these molecules to ADPR. In an experiment (see figure 2H) the ADPR competitor 8-Br-ADPR was used. Application of 100 µM of 8-Br-ADPR upon challenging the cells with 10 µM cADPR, together with 100 µM 8-Br-ADPR, significantly suppressed the action of cADPR (see figure 2G). Experiments using primary beta cells of transgenic mice deficient in CD38 expression further strengthened this. Consistent with this was the fact that, like ADPR, both NAD\(^{+}\)- and cADPR effects were mediated through P2Y and adenosine receptors, since the combined suppression of these receptors by suramin and CGS-15943 completely antagonizes the response (Fig. 2I and Fig. 2H, respectively). Furthermore, ADPR and cADPR show a similar pharmacological profile, since suramin is more effective than CGS-15943 in suppressing the response to cADPR (Fig. 2H).
Figure 2: TRPM2 channels, purinergic and adenosine receptors are activated by ADPR in rat INS-1 beta cells. (A) Averaged development of TRPM2 currents assessed by whole cell patch-clamp measurements in INS-1 cells. Cells were perfused with either 100 μM ADPR (open circles, n = 11) or 100 μM ADPR + 1 mM AMP (closed circles, n = 9). Current amplitudes were assessed at ~80 mV, normalized for cell size, averaged and plotted versus time of the experiment. The standard voltage-protocol was ramping from ~100 mV to +100 mV over 50 ms and acquired at 0.5 Hz. Holding potential was 0 mV. Error bars indicated S.E.M. (B) Typical current-voltage (I/V) relationship of currents evoked by 1 mM ADPR (black trace), 100 μM ADPR (blue trace) or 100 μM ADPR + 1 mM AMP (red trace) taken from example cells and extracted at 100 s into the experiment. (C) Dose-response behavior of TRPM2 currents measured in INS-1 to increasing internal ADPR concentrations. Current amplitudes were measured at ~80 mV, averaged, normalized to cell size and plotted against the respective ADPR concentration (n = 5 to 11). A dose-response fit to the data resulted in a K<sub>D</sub> value of 110 μM with a Hill coefficient of 1. (D) Averaged Ca<sup>2+</sup> signal measured in intact Fura-2 AM loaded INS-1 cells in response to increasing concentrations of extracellular ADPR and in the absence of extracellular Ca<sup>2+</sup> (100 nM (black trace, n = 6), 1 μM (red trace, n = 6), 10 μM (blue trace, n = 6), 30 μM (green trace, n = 6)). (E) Averaged Ca<sup>2+</sup> signal measured in intact Fura-2 AM loaded INS-1 cells in response to 30 μM extracellular ADPR (black trace, control, n = 6) or 100 μM ADPR in the absence of extracellular Ca<sup>2+</sup> and in the presence of either 100 μM suramin (green trace, n = 6), 1 μM CGS-15943 (blue trace, n = 11) or both 100 μM suramin and 1 μM CGS-15943 (red trace, n = 6) in the bath solution. (F) Averaged Ca<sup>2+</sup> signal measured in intact Fura-2 AM loaded INS-1 cells in response to 10 μM ADPR in the absence of extracellular Ca<sup>2+</sup> and in the presence of either 100 μM suramin (black trace, n = 6) or 1 μM CGS-15943 (red trace, n = 6) in the external solution. (G) Averaged Ca<sup>2+</sup> signal measured in intact Fura-2 AM loaded INS-1 cells in response to increasing concentrations of extracellular cADPR and in the absence of extracellular Ca<sup>2+</sup> (1 μM (black trace, n = 5), 10 μM (blue trace, n = 8), 30 μM (green trace, n = 5)). The red trace is the average response of 4 cells to application of 1 mM NAD<sup>+</sup>. Note that only 1 cell out of 4 responded with a Ca<sup>2+</sup> signal at all. Application start as indicated by the arrow. (H) Averaged Ca<sup>2+</sup> signal measured in intact Fura-2 AM loaded INS-1 cells in response to application of 100 μM cADPR in the absence of extracellular Ca<sup>2+</sup> and in the presence of
either 100 µM suramin (blue trace, n = 6) or 1 µM CGS-15943 (black trace, n = 5) or both suramin and CGS-15943 (green trace, n = 6) in the external solution. Green trace 100 µM 8-Br-ADPR + 10 µM cADPR n = 12 (I) Average Ca\(^{2+}\) signal measured in intact Fura-2 AM loaded INS-1 cells in response to application of 10 µM (black trace, n = 5), 30 µM (blue trace, n = 7) or 100 µM (green trace, n = 10) NAD\(^+\) in the absence of extracellular Ca\(^{2+}\). The red trace indicates application of 100 µM NAD\(^+\) in the presence of 100 µM suramin and 1 µM CGS-15943 (n = 7) in the external solution.

**Effects of intracellular ADPR and pharmacological characterization of stores in INS-1 cells**

After evaluating the effects of extracellular ADPR causing Ca\(^{2+}\) release through known G-Protein-coupled receptors involving pathways, the possibility was considered, that ADPR could act intracellularly as a second messenger causing calcium release. This was tested by intracellular perfusion of ADPR into INS-1 cells. As illustrated in Fig. 3A, intracellular perfusion of cells with different concentrations of ADPR in the absence of extracellular Ca\(^{2+}\) and presence of suramin and CGS-15943 in the bath, produced a robust and sizeable increase in [Ca\(^{2+}\)], in a concentration-dependent manner, suggesting that ADPR indeed causes Ca\(^{2+}\) release from intracellular stores. Since TRPM2 is a downstream target of reactive oxygen species\(^6\) it was further tested whether hydrogen peroxide (H\(_2\)O\(_2\)) could mediate Ca\(^{2+}\) release in these cells. As illustrated in Fig. 3B, perfusing cells with 100 µM H\(_2\)O\(_2\) in the presence of heparin to suppress IP\(_3\) receptors indeed evoked Ca\(^{2+}\) release, consistent with its ability to activate TRPM2. It was confirmed that the ADPR-mediated responses involved TRPM2 by molecular knock-down of TRPM2 by siRNA. As shown in Fig 3C, TRPM2-specific siRNA, but not a scrambled control siRNA, largely suppressed ADPR-induced Ca\(^{2+}\) release (Fig. 3C). The efficacy of specific siRNA knock-down of TRPM2 was established by monitoring TRPM2 channel activity in the plasma membrane. Out of three siRNA duplex oligoribonucleotides (25-mers) targeting rat TRPM2 the most efficient sequence was screened by patch-clamp analysis. This confirmed, that siRNA treatment resulted in nearly complete suppression of functional channels, since ADPR-induced membrane currents were strongly suppressed (Fig. 3D). In addition to the functional data, the peripheral and intracellular localization of TRPM2 was confirmed by immunofluorescence (Fig. 3E). Interestingly, these data show that TRPM2 very rarely, if at all, co-localizes with the endoplasmic reticulum (ER; Fig. 3E, upper and lower right panels). Instead, TRPM2 shows a punctuate distribution throughout the intracellular
compartment (Fig. 3A, lower left and right panels), possibly reflecting localization in a vesicular compartment. The effect of internal ADPR was further characterized by the use of commonly known pharmacological tools influencing calcium homeostasis (Figure 3F). Co-perfusion of 100 ADPR with 100 µg/ml heparin did not affect the response, whereas 20 µM ryanodine both in patch pipette and bath seemed to lower the calcium release signal (Figure 3F). This might be due to the nature of this compound in acting as a calcium release agonist at low- and as an inhibitor at high concentration. Preincubation with the SERCA (sarco/endoplasmic- reticulum calcium ATPase) inhibitor Thapsigargin (500 nM) clearly showed that the effect of ADPR in elevating levels of calcium was lost (Figure 3F), indicating that TRPM2 is located in thapsigargin sensitive stores. Together, these data demonstrate that TRPM2 proteins in INS-1 beta cells, as in the heterologous overexpression system, function as both Ca$^{2+}$-permeable cation channels in the plasma membrane and as Ca$^{2+}$ release channels in intracellular stores.
Figure 3: TRPM2 functions as calcium release channel in INS-1 beta cells. (A) The panel shows balanced Fura-2 experiments. Averaged Ca\(^{2+}\) signal in whole cell patch-clamped INS-1 cells preloaded with Fura-2 AM. Whole-cell break-in was at the time indicated by the red arrow. Cells were kept in 0 Ca\(^{2+}\) external solution approximately 30 seconds previous to brake in and perfused with internal solution containing 100 µM ADPR (black trace, n = 11) 30 µM ADPR (blue trace, n = 9) and 10 µM ADPR (red trace, n = 6) including 200 µM Fura-2. Cells were preincubated in 100 µM suramin and 1 µM CGS-15943. (B) Using the balanced Fura-2 approach, the graph shows averaged Ca\(^{2+}\) signal in whole cell patch-clamped INS-1 cells preloaded with Fura-2 AM. Whole-cell break-in was at the time indicated by the red arrow. Cells were kept in 0 Ca\(^{2+}\) external solution and perfused with 100 µM H\(_2\)O\(_2\) (black trace, n = 10) and 200 µM Fura-2. (C) Balanced Fura-2 approach, showing averaged Ca\(^{2+}\) signal in whole cell patch-clamped INS-1 cells preloaded with Fura-2 AM. The black trace represents Ca\(^{2+}\) signals from cells treated with scramble control siRNA (n = 10). The red trace is the response measured in cells treated with TRPM2-specific siRNA (n = 10). Cells were kept in 0 Ca\(^{2+}\) external solution supplemented with 100 µM suramin and 1 µM CGS-15943. The internal solution contained 100 µM ADPR and 200 µM Fura-2. Whole-cell break-in was at the time indicated by the red arrow. (D) Averaged development of TRPM2 currents assessed by whole cell patch clamp measurements in INS-1 cells treated with scramble control siRNA (closed circles, n = 8) or TRPM2-specific siRNA (open circles, n = 14).
Currents were analyzed as described in Fig. 2A. (E) Detection and cellular localization of TRPM2 by immunofluorescence. An anti-mouse TRPM2 serum specifically recognizes a protein in INS-1 cells with cytosolic as well as plasma membrane (left upper panel, green) distribution. Intracellular TRPM2 label is largely excluded from the endoplasmic reticulum (ER, middle upper panel, red) network, as evidenced by the merged image (right upper panel, note absence of significant yellow spots). DAPI was used as a nuclear counterstain (blue). The white rectangle indicates the area of expanded view depicted in the respective lower panels. Note the punctuated appearance of intracellularly located TRPM2, indicating vesicular localization. Cells were visualized using ApoTome Axiovert 200 imaging microscope an Axiocam MRM CCD camera and the Zeiss AxioVision software. Images of cells that are representative of the entire population are shown (63x magnification). The staining was gratefully provided by Prof. Dr. Santiago Partida-Sanchez (F) Same experimental conditions as in A. Cells were perfused with 100 µM ADPR in addition with 100 µg/ml heparin (black trace, n = 7). Blue trace shows 20 µM ryanodine both in bath and patch pipette (n = 6). Cells were preincubated with 500 nM thapsigargin (red trace, n = 9).

**TRPM2 in primary mouse beta cells**

Although the INS-1 cell line is considered an excellent model for pancreatic beta cells, cell lines often do not fully reflect the properties of primary cells. Therefore the analysis was extended to primary pancreatic beta cells, isolated from C57BL/6 mice. Similar sets of experiments as those described above were performed on these cells.

**Extracellular ADPR acts on P2Y receptors**

First, the potency of the agonist ADPR to activate TRPM2-like currents in the plasma membrane was evaluated. Experiments were performed 24-72 hours after isolation of pancreatic beta cells from C57BL/6 mice. Cells were kept under the same conditions as INS-1 cells and subjected to the same experimental protocols with identical ionic compositions of internal and external solutions. Cells were perfused with various concentrations of ADPR in Cs-glutamate-based pipette solutions to reveal TRPM2 currents while suppressing any contaminating K⁺ currents. Under these conditions, ADPR induced rapid activation of a linear current showing the typical characteristics of TRPM2 (Fig. 4B). Activation kinetics of these currents were similar to the ones obtained in INS-1 cell lines, reaching peak amplitudes within 30-50 seconds (Fig 4A). The ADPR-induced currents were concentration dependent with an EC₅₀ of ~360 µM ADPR (Fig. 4C), i.e., roughly 3-fold higher than in INS-1 cells and the maximal current densities in primary beta cells were about ~80 pA/pF at ~80 mV (Fig. 4C), which is similar to the current densities observed in INS-1 cells. Experiments using cells isolated from TRPM2 knock-out mice did elicit TRPM2 currents. Perfusion of beta cells from TRPM2-deficient mice with concentrations of 1 mM ADPR failed to evoke any membrane currents (red trace figure 4A) and current-voltage relationship remained essentially flat (figure 4B red
trace). Hence, it was clear that TRPM2 is expressed as a functional ion channel in primary beta cells where it can be activated by intracellular ADPR.

**Intracellular ADPR mediates calcium release through TRPM2**

In addition to its function as a plasma membrane channel, TRPM2 in primary beta cells, like in the INS-1 cell line, is capable of mediating Ca$^{2+}$ release from intracellular stores. Figure 4I illustrates that intracellular perfusion of wild-type mouse beta cells with 300 µM ADPR, a concentration that activates TRPM2 channels in the plasma membrane (see Fig. 4C), evokes Ca$^{2+}$ release transients, whereas the same concentration fails mobilize calcium in cells obtained from TRPM2 knock-out mice.

**cADPR causes calcium release in beta cells**

In additional experiments, the extracellular effects of ADPR, NAD$^+$, and cADPR were investigated in primary beta cells. In these experiments, the cells responded similarly to these compounds as INS-1 cells in that all three agonists produced clear Ca$^{2+}$-release transients when applied to intact cells. The potency of ADPR was similar to that of HEK293 cells, with a threshold concentration of ~100 µM ADPR. In contrast to the rat insulinoma cell line INS-1, CGS-15943 had no effect on the response, whereas suramin alone at 100 µM completely abolished extracellularly-mediated ADPR effects (Fig. 4E), suggesting that primary mouse beta cells lack adenosine receptors. Although mouse beta cells express CD38$^{96}$, NAD$^+$ did not elicit any response even at 1 mM (Fig. 4F). This result might be explained by the ability of NAD$^+$ to act as an inhibitor of the plasma membrane P2Y receptors in this cascade. Co-application of 100 µM NAD$^+$ with 100 µM of ADPR completely prevented the release of calcium, and lower concentration of 30 µM NAD$^+$ showed a reduced effect (FIG. 4H). Similar observations were made in HEK293 cells where receptor distribution in terms of ADPR action appears to be similar as in primary beta cells. Higher concentrations of NAD$^+$ (1 mM) were necessary to abolish the action of 100 µM ADPR (Data not shown). However, cADPR was effective and its threshold concentration was ~300 µM, similar in relative terms to the 10-fold higher threshold observed in INS-1 cells. Since mouse beta cells express CD38, the CD38 knock-out mouse$^{81}$ was used to examine whether the efficacy of cADPR relies on the presence of this enzyme. The experiments illustrated in Fig. 4G demonstrate that this is the case, since CD38-deficient beta cells no longer respond to cADPR, but retain the
responsiveness to P2Y receptor stimulation. CD38 knock-out cells are still capable of mediating signals induced by either 300 μM ADPR or 100 μM ATP, indicating no functional down regulation of nucleotide-sensitive receptors in cells isolated from knock-out mice.

Figure 4: ADPR activates purinergic receptors and causes calcium influx and release through TRPM2 channels in mouse pancreatic beta cells. (A) Averaged development of TRPM2 currents assessed by whole cell patch-clamp measurements in wild-type and TRPM2 knock-out mouse pancreatic beta cells. Wild-type cells were perfused with either 300 μM ADPR (open circles, n = 7), 300 μM ADPR + 1 mM AMP (closed circles, n = 9) or 3 mM ADPR (n = 6). Knock-out mouse cells were perfused with 1 mM ADPR (red trace, n = 6). Current amplitudes were assessed as described in Fig. 2A. Error bars indicated S.E.M. (B) Typical current-voltage (I/V) relationship of currents evoked by 1 mM ADPR (black trace), 300 μM ADPR (blue trace) or 300 μM ADPR + 1 mM AMP (green trace) taken from example wild-type cells and extracted at 100 s into the experiment. Red trace trpm2−/− mouse taken at 100 seconds perfused with 1 mM ADPR (C) Dose-response behavior of TRPM2 currents measured in mouse beta cells to increasing internal ADPR concentrations. Current amplitudes were measured at −80 mV, averaged, normalized to cell size and plotted against the respective ADPR concentration (n = 5 to 7). A dose-response fit to the data resulted in an EC50 value of 360 μM with a Hill coefficient of 1. (D) Averaged Ca2+ signal measured in intact Fura-2 AM loaded mouse beta cells in response to increasing concentrations of extracellular ADPR and in the absence of extracellular Ca2+ (1 μM (red trace, n = 4), 10 μM (blue trace, n = 5), 30 μM (green trace, n = 6), 100 μM (black trace, n = 6)). Start of application as indicated by black arrow. (E) Averaged Ca2+ signal measured in intact Fura-2 AM loaded mouse beta cells in response to application of 200 μM ADPR in the absence of extracellular Ca2+ and in the presence of either 100 μM
suramin (red trace, n = 6) or 1 μM CGS-15943 (black trace, n = 8) in the external solution. (F) Averaged Ca²⁺ signal measured in intact Fura-2 AM loaded mouse beta cells in response to application of either 100 µM cADPR (red trace hidden behind blue trace, n = 5), 300 µM cADPR (black trace, n = 6) or 1 mM NAD⁺ (blue trace, n = 8). (G) Averaged Ca²⁺ signal measured in intact Fura-2 AM loaded pancreatic beta cells isolated from CD38 knock-out mice in response to application of either 100 µM ADPR (black trace, n = 4), 300 µM cADPR (red trace, n = 20) or 100 µM ATP (blue trace, n = 8). (H) Averaged Ca²⁺ signal measured in intact Fura-2 AM loaded mouse beta cells in response to 100 µM ADPR with either 30 µM β-NAD (black trace, n = 6) or 100 µM β-NAD (red trace, n = 6) in the absence of extracellular Ca²⁺. Start of application as indicated by black arrow (I) The graph shows balanced Fura-2 experiments. Averaged Ca²⁺ signal in whole cell patch-clamped mouse pancreatic beta cells preloaded with Fura-2 AM. Whole-cell break-in was at the time indicated by the red arrow. Cells were kept in 0 Ca²⁺ external solution containing 100 µM suramin and perfused with internal solution containing 300 µM ADPR both wild-type (thick black trace, n = 7) and TRPM2 knock-out (red trace, n = 10) with 200 µM Fura-2. The gray traces exemplify two representative responses measured in individual wild-type cells.

**TRPM2 function is limited to calcium release in mouse dendritic cells**

To further investigate the novel finding of TRPM2 acting both as a calcium influx and release channel, it was tested whether this might apply to other cell systems. Preliminary data from a cloned murine dendritic cell line DC 2.4 indicated TRPM2 being functionally present in the plasma membrane (data not shown). In collaboration with the group of Santiago Partida-Sanchez, experiments were performed in mouse bone marrow-derived dendritic cells. In contrast to the cell line, neither high concentrations of up to 1 mM ADPR nor direct perfusion of 100 µM H₂O₂ elicited a linear current exhibiting the typical characteristics of TRPM2 (Fig. 5A). Even after 150 seconds no activation developed over time (Fig. 5B), suggesting that TRPM2 was either absent or non-functional in the plasma membrane. Interestingly, the ability of ADPR in mobilizing calcium from internal stores was almost two orders of magnitude more efficient than in the rat pancreatic cell line INS-1 (see Fig. 3A and 5C). Cells were perfused with various concentrations of ADPR ranging from 100 nM to 1 mM. Even at low concentrations of 1 µM a distinct calcium transient was resolved (Fig. 5C). The release of calcium by 10 µM ADPR was completely inhibited by the TRPM2 antagonist AMP (100 µM) as well as the novel bromine-substituted derivative of ADPR 8-Bromo-ADPR (100 µM)⁷⁰. Dendritic cells derived from mice deficient of TRPM2 failed to produce calcium release even at 1 mM ADPR. In addition, immuno-staining of intact cells hinted at a possible localization of protein in intracellular compartments (see figure 5D), although co-staining with adequate markers to identify these organelles remains to be analyzed.
Figure 5: ADPR fails to activate TRPM2-like currents in bone marrow-derived mouse dendritic cells, but induced calcium release. (A) Current-voltage (I/V) relationship evoked by 1 mM ADPR (blue trace), 100 µM H₂O₂ (black trace) taken from example cells and extracted at 100 s into the experiment. (B) Averaged development of currents assessed by whole cell patch-clamp measurements in mouse dendritic cells. Cells were perfused with either 1 mM and 100 µM ADPR (open circles, n = 2, closed circles n=9) or 100 µM H₂O₂ (closed square, n = 5) or 3 mM ADPR (n =6). Current amplitudes were assessed as described in Fig. 2A. Error bars indicated S.E.M. (C) The graph shows balanced Fura-2 experiments. Averaged Ca²⁺ signal in whole cell patch-clamped mouse dendritic cells preloaded with Fura-2 AM. Whole-cell break-in was at the time indicated by the red arrow. Cells were kept in 0 Ca²⁺ external solution and perfused with internal solution containing different concentrations of ADPR between 100 nM and 1 mM (thick black trace 10 µM, 100 µM and 1 mM, grey trace 1 µM, blue 100 nM n = 5-8) and 200 µM Fura-2. In Traces yellow and green cells were perfused with ADPR in addition with 8-Br-ADPR and 100 µM AMP (yellow and green 10 µM ADPR in addition with either 100 µM 8-Br-ADPR or 100 µM AMP). TRPM2 knock-out mouse was perfused with 1 mM ADPR (red trace) (D) Immunostaining of bone marrow-derived dendritic cells treated with polyclonal rabbit anti human TRPM2 antibody (right picture, green). Nuclei are labeled by DAPI (left picture, blue). The staining was greatfully provided by Dr. Adriana Sumoza-Toledo.
Synergistic regulation of endogenous TRPM2 channels by adenine dinucleotides in primary human neutrophils

Regulation of TRPM2 by intracellular Ca$^{2+}$

Neutrophils in general have been known to highly express TRPM2$^{97}$. Further experiments investigating channel-gating mechanisms by different ribosylated nucleotides were carried out using primary neutrophils isolated from freshly drawn human blood. It had been reported previously that cADPR, H$_2$O$_2$, and NAADP can synergize with the primary agonist ADPR to more efficiently activate TRPM2$^{63}$. Recently, it was demonstrated that intracellular cations can affect the sensitivity of TRPM2 channels to ADPR and cADPR in Jurkat T cells and HEK293 cells overexpressing TRPM2$^{63, 64}$. This might also influence the synergistic effects of other TRPM2 modulators. Detailed analyses of the facilitatory actions of cADPR, H$_2$O$_2$ and NAADP in relation to ADPR-induced TRPM2 activation in primary human neutrophils were conducted using K$^+$-based solutions and assessing agonist effects over a large concentration range.

An ADPR dose-response curve in neutrophils isolated from whole human blood was established. Figure 6A shows the average normalized time course of inward currents measured in neutrophils at −80 mV and evoked by increasing concentrations of intracellular ADPR (100 nM – 1 mM) added to the standard K$^+$-glutamate based pipette solution. The intracellular calcium concentration ([Ca$^{2+}$]$_i$) was left unbuffered by omission of any exogenous Ca$^{2+}$ chelators, since clamping [Ca$^{2+}$]$_i$ to about zero with 10 mM BAPTA prevented activation of TRPM2 even in the presence of 1 mM ADPR (data not shown). This caused a dose-dependent activation of ADPR-dependent currents that showed typical properties of TRPM2 with a linear current-voltage (IV) relationship and a reversal potential ($E_{rev}$) of 0 mV (Fig. 6C). To establish the dose-response curve of ADPR-induced currents, the maximum ADPR-evoked currents measured at 100 s into the experiment were extracted, averaged, normalized to cell size and plotted versus their respective ADPR concentration (Fig. 6D; black circles, n = 4-10). A dose-response fit to the data yielded a half-maximal effective concentration (EC$_{50}$) for ADPR of 1.1 µM with a Hill coefficient of 1.5. This was considerably lower than the EC$_{50}$ values obtained for heterologously expressed TRPM2 in HEK293 cells (10 µM)$^{63, 98}$, or native TRPM2 in Jurkat T cells (7 µM)$^{64}$, U937 monocytes (40 µM)$^{21}$, and RINm5f cells (20 µM,
unpublished observations), indicating that both ionic and cellular environment can determine the sensitivity of TRPM2 channels to ADPR.

Several cellular systems have been shown to require the presence of intracellular and/or extracellular Ca\(^{2+}\) to evoke ADPR-induced TRPM2 currents, including human neutrophils \(^{21, 86, 98-100}\). When perfusing human neutrophils with a fixed concentrations of 1 mM ADPR in the presence of increasing intracellular Ca\(^{2+}\) concentrations ranging from 0 to 1 \(\mu\)M (Fig. 6B; \(n = 5-9\)), TRPM2 currents reached peak current amplitude faster than in unbuffered conditions (see Fig. 6A), within 10-20 s. Typically, these currents also inactivated by about 20% within the time frame of the experiments. Fitting a dose-response curve to the average normalized currents measured at the peak showed that the Ca\(^{2+}\) concentration required for half-maximal activation of TRPM2 at 1 mM ADPR was 300 nM with a Hill coefficient of 2 (Fig. 6D; red circles, \(n = 5-9\)).

ADPR-induced activation of TRPM2 currents are negatively regulated by increasing intracellular AMP concentrations in Jurkat T cells and HEK293 cells overexpressing the channel \(^{63, 64}\). However, lower concentrations of AMP (5 \(\mu\)M) reportedly failed to inhibit TRPM2 in neutrophils activated by saturating ADPR concentrations (5 \(\mu\)M) in intracellular Cs\(^+\) conditions \(^{86}\). Therefore, the inhibitory action of AMP was evaluated at 1 \(\mu\)M ADPR, the EC\(_{50}\) for this second messenger in neutrophils. As can be seen in Fig. 6E, 100 \(\mu\)M AMP substantially suppressed the activation of TRPM2 currents, indicating that AMP has the potential to inhibit ADPR-mediated TRPM2 activity when produced in excess of ADPR. To specify this effect, a complete inhibitory concentration-response curve with various AMP concentrations between 1 \(\mu\)M and 300 \(\mu\)M in the presence of 1 \(\mu\)M ADPR was constructed. This revealed an IC\(_{50}\) for AMP of 10 \(\mu\)M with a Hill coefficient of 2 (Fig. 6F). In summary, the results presented show that ADPR activates TRPM2 currents very effectively in a dose-dependent manner in primary human neutrophils. Furthermore, this activation is dependent on and facilitated by intracellular Ca\(^{2+}\) and counteracted by AMP. Interestingly though, in contrast to the dendritic cells, ADPR could not trigger calcium release. Cells that were loaded with Fura-2-AM and perfused with 10 \(\mu\)M ADPR and 200 \(\mu\)M Fura-2 (balanced Fura) could not elevate intracellular calcium levels in the absence of extracellular calcium (Fig. 6G \(n = 6\)). Results from HPLC analysis indicated that the basal ADPR concentration of human...
neutrophils is around 5 μM and this value is not significantly altered by fMLP-induced receptor stimulation \(^{86}\). ADPR-induced TRPM2 activation required intracellular calcium levels higher than 100 nM (Fig. 6B) and the EC\(_{50}\) for TRPM2 activation was around 1 μM in the presence of Ca\(^{2+}\) (Fig. 1D and \(^{86}\)). Therefore, it was reasoned that increasing the intracellular calcium concentration alone should activate TRPM2 current in a perforated-patch situation, where cellular ADPR levels would be left unperturbed. Combined perforated-patch and balanced Fura-2 experiments were performed, where cells were first preloaded with Fura-2-AM and subsequently patched with the standard intracellular solution containing 300 μM amphotericin B and 200 μM Fura-2 (see methods). The pipette potential was kept at 0 mV. Upon establishment of the perforated-patch (Rs < 20 MΩ), the standard voltage ramp was started (see methods) and ionomycin (2 μM) was applied 20 s thereafter for 5 s (Fig. 6H). While this induced a rapid increase of intracellular calcium of around 300 nM (Fig. 6F lower trace), it did not cause a concomitant activation of TRPM2 currents as observed over a time span of 200 s (Fig. 6H, upper trace). This indicated that either the local calcium concentration in the vicinity of TRPM2 channels did not reflect the global calcium increase or, alternatively, resting ADPR concentration were lower than sufficient for synergistic effect in concert with calcium. ADPR-activated single channel conductance of TRPM2 reportedly is between 56 pS and 67 pS, exhibiting characteristically long open times of up to tenths of seconds \(^{21,64,97,99}\). In neutrophils, application of 300 μM ADPR evoked a 56 pS single channel conductance typical for TRPM2 in excised inside-out patches at potentials negative to 0 mV \(^{97}\). To evaluate TRPM2 single channel conductance at potentials positive to 0 mV, whole-cell experiments were performed where neutrophils were perfused with threshold concentrations of ADPR to evoke isolated activity of individual channels and using a ramp protocol spanning −100 mV to +100 mV over 20 s. While 100 nM ADPR perfusion caused activity of two channels in 2 out of 6 cells (data not shown), increasing ADPR to 200 nM reliably activated two to five channels in 6 out of 6 cells, as assessed over the time course of the experiment. Figure 6I shows four consecutive ramp measurements of TRPM2 single-channel activity recorded in a representative cell directly after whole-cell establishment (Fig. 6I, first trace at 0 s) and at 22 s, 44 s and 66 s into the experiment. Activity of two channels can be seen, with second-long open times, a reversal potential of
0 mV and a slight outward rectification. A line fit to potentials negative to 0 mV gave a single-channel conductance of $43 \pm 0.4$ pS ($n = 3$), whereas a fit to potentials positive to 0 mV showed a single-channel conductance of $63 \pm 2$ pS ($n = 3$). Since Jurkat T lymphocytes represent a well-known model to study T-cell physiology, it was assumed, that primary T lymphocytes isolated from whole human blood would also show ADPR-induced TRPM2 currents. However, when perfusing human naïve T lymphocytes from peripheral blood with 1 mM ADPR in standard K$^+$-glutamate based and Ca$^{2+}$-unbuffered solution, TRPM2-like currents were never observed (Data Fig. 6J; open circles, $n = 10$). In summary, the results presented in Figure 6 show that ADPR activates TRPM2 currents very effectively in a dose-dependent manner in primary human neutrophils. Furthermore, this activation is dependent on and facilitated by intracellular Ca$^{2+}$ and counteracted by AMP.
Figure 6: Ca\(^{2+}\) facilitates activation of TRPM2 currents in the presence of ADPR. (A) Average normalized TRPM2 currents activated by ADPR in human neutrophils. Currents were measured with a voltage ramp from –100 mV to +100 mV over 50 ms at 0.5 Hz intervals from a holding potential of 0 mV. Inward current amplitudes were extracted at –80 mV, averaged and plotted versus time. Cells were perfused with the standard intracellular K\(^+\)-based solution in the absence of exogenous Ca\(^{2+}\) buffers and supplemented with increasing ADPR concentrations as indicated (n = 4-10). Standard extracellular solution contained 1 mM Ca\(^{2+}\). (B) Average normalized TRPM2 currents activated by 1 mM ADPR and variable intracellular Ca\(^{2+}\) concentrations as indicated (n = 5-9). Currents were analyzed as in (A). (C) Current-voltage (I/V) curves taken from representative cells perfused with 30 µM ADPR. (D) Dose-response curves of ADPR-induced TRPM2 currents in unbuffered internal solution (black circles, n = 4-10) and in clamped Ca\(^{2+}\) solutions at 1 mM fixed ADPR (red circles, n = 5-9). Data were plotted against ADPR or Ca\(^{2+}\) concentrations and fitted with dose-response curves. The EC\(_{50}\) values are indicated in the panel. Hill coefficients were 1.5 for unbuffered and 2 for clamped Ca\(^{2+}\). Data were acquired as described in (A). To establish the dose-response curves, the peak inward currents at –80 mV were extracted, averaged and plotted versus the respective ADPR or Ca\(^{2+}\) concentration. (E) Average normalized TRPM2 inward currents at –80 mV evoked by 1 µM ADPR in the absence (black circles, n = 9) or presence of 100 µM AMP (red circles, n = 6). Error bars represent S.E.M. (F) The panel depicts the inhibitory dose-response
Regulation of TRPM2 by cADPR and \(\text{H}_2\text{O}_2\)

TRPM2 currents in overexpressing HEK293 cells and Jurkat T lymphocytes can be activated by perfusion of cells with increasing cADPR concentrations, albeit at a significantly lower efficiency and reduced amplitude compared to ADPR, unless those two agents are co-perfused and synergize \(^{63,64}\). Since a previous report questioned the ability of cADPR to activate or synergize with native TRPM2 in human neutrophils \(^{86}\), this issue was addressed by performing a detailed dose-response analysis of TRPM2 currents evoked by increasing cADPR concentrations between 300 nM and 1 mM added to the standard K\(^{+}\)-glutamate internal solution (Fig. 7A). The average normalized maximum currents measured at 100 s were plotted against the respective cADPR concentration. The data were fitted with a dose-response curve. This established that cADPR activates TRPM2 currents with an \(\text{EC}_{50}\) of 44 \(\mu\)M and a Hill coefficient of 1 (Fig. 7D, blue circles, \(n = 4\)-9). This \(\text{EC}_{50}\) was shifted about 15-fold to the left by merely adding a subthreshold concentration of 100 nM ADPR to the respective cADPR concentrations. This is shown in Fig. 7B, which plots the average normalized time course of TRPM2 activation evoked by perfusing cells with the standard K\(^{+}\)-glutamate solution supplemented with 100 nM ADPR and increasing cADPR concentrations varying between 100 nM and 100 \(\mu\)M. Whereas 1 mM of cADPR was needed to fully activate TRPM2 currents, only 100 \(\mu\)M cADPR was required in the presence of subthreshold 100
nM ADPR (Fig. 7A and Fig. 7B). Fig. 7 C illustrates I/V curves extracted at 100 s from representative neutrophils perfused with either 1 mM cADPR (black trace), 3 µM cADPR (blue trace) or a combination of 100 nM ADPR plus 3 µM cADPR (red trace). Plotting the average normalized current evoked by 100 nM ADPR plus increasing cADPR against the respective cADPR concentration resulted in a dose-response curve whose fit yielded an EC₅₀ of 3 µM with a Hill coefficient of 2 (Fig. 2D, red circles, n = 5-7). This demonstrates that cADPR is able to enhance the effectiveness of subthreshold ADPR levels to efficacy levels that are almost comparable to ADPR-induced TRPM2 activation (EC₅₀ = 1.1 µM, see Fig. 6C and 7D).

It has previously been shown that H₂O₂ activates TRPM2 currents, although in a limited fashion⁶²,¹⁰¹,¹⁰². This suggests that the effects of H₂O₂ on TRPM2 may be very similar to those of cADPR, in that both compounds are able to potentiate ADPR-induced TRPM2 activation.⁶³ Therefore the question was revisited whether H₂O₂ could facilitate ADPR-induced TRPM2 currents in human neutrophils. Cells perfused with the standard K⁺-glutamate-based solution supplemented with either 100 µM H₂O₂ (Fig. 7E, open squares, n = 6) or 100 nM ADPR (Fig. 7E, open circles (behind open squares), n = 6). Neither of these manipulations activated any significant TRPM2 currents within the observed time frame of the experiment. However, co-perfusing the cells with 100 µM H₂O₂ and 100 nM ADPR was effective in activating TRPM2 (Fig. 7E, closed circles, n = 6). It was assessed whether cADPR-induced TRPM2 currents could be blocked by the competitive inhibitor 8-Bromo-cADPR, as it had been reported previously for heterologously expressed TRPM2 in HEK293 cells and Jurkat T lymphocytes.⁶³,⁶⁴ Indeed, co-perfusion of 30 µM cADPR and 100 µM 8-Bromo-cADPR completely suppressed activation of TRPM2 currents (Fig. 7F, red circles, n = 6). These data confirm that both the synergy of cADPR and H₂O₂ with ADPR and the antagonistic effect of 8-Bromo-cADPR on cADPR are present in primary human neutrophils, even at lower concentrations and thus with higher potency and efficacy.
Figure 7: ADPR and cADPR synergize and 8-Bromo-cADPR inhibits TRPM2 currents. (A) Average normalized TRPM2 currents activated by cADPR in human neutrophils (n = 4–9). Intracellular conditions and data acquisition/analysis were as in Fig. 1A. (B) Average normalized TRPM2 currents activated by various cADPR concentrations in the presence of 100 nM ADPR (n = 5–7). Intracellular conditions and data acquisition/analysis as in (A). (C) I/V curves taken from representative cells perfused with 1 mM cADPR (black trace), 3 µM ADPR (blue trace) or 3 µM cADPR + 100 nM ADPR (red trace). (D) Dose-response curves of TRPM2 currents evoked by ADPR (black circles, same data set as in Fig. 1), cADPR (blue circles, n = 4–9) or subthreshold ADPR (100 nM) + increasing cADPR concentrations (red circles, n = 5–7) in unbuffered K⁺-based internal solution. Current amplitudes were plotted against concentrations and fitted with dose-response curves. The EC₅₀ values are indicated in the panel. Hill coefficients were 1 for cADPR and 2 for cADPR with 100 nM ADPR. To establish the dose-response curves, the peak inward currents at −80 mV were extracted, averaged and plotted versus the respective ADPR or cADPR concentration. (E) Average normalized TRPM2 inward currents at −80 mV evoked by 100 nM ADPR and in the absence (open circles, n = 6) or presence of 100 µM H₂O₂ in the patch pipette (closed circles, n = 6). 100 µM H₂O₂ in the pipette without any ADPR did not evoke any currents (open squares, n = 6). Note that the ADPR-only data are overlapping with the H₂O₂ time course and are hidden from view. Error bars represent S.E.M. (F) Average normalized TRPM2 inward currents at −80 mV evoked by 30 µM cADPR and in the absence (black circles, n = 7) or presence of 100 µM 8-Br-cADPR (red circles, n = 6). Error bars represent S.E.M.

Regulation of TRPM2 by NAADP

NAADP has gained significant interest as a potent Ca²⁺-release agonist, acting at low nanomolar concentrations. It had been previously reported that NAADP potentially can activate TRPM2 in Jurkat T cells and HEK293 cells overexpressing the channel, albeit in the high µM concentration range. In addition, it was demonstrated for the TRPM2-overexpression system that NAADP synergizes with ADPR in the low micromolar range. TRPM2 activation in neutrophils is significantly more sensitive to both ADPR and cADPR stimulation. Therefore, it was investigated whether this would
hold true for the recruitment of the current by intracellular NAADP. Human neutrophils were perfused with the standard K⁺-glutamate-based internal solution supplemented with increasing NAADP concentrations ranging between 3 µM – 1 mM. This resulted in a dose-dependent activation of currents (Fig. 8A) that showed the typical I/V behavior of TRPM2 (Fig. 8C, black trace). A fit to the maximum normalized currents extracted at 100 s into the experiment resulted in an EC₅₀ of 95 µM and a Hill coefficient of 1.6 (Fig. 8D, black circles, n = 3-5), slightly less efficient than cADPR. Interestingly, when co-perfusing cells with a subthreshold ADPR concentration of 100 nM and increasing NAADP (Fig. 8B), TRPM2 activation was only facilitated at or below 30 µM NAADP, whereas NAADP levels above 30 µM failed to do so and the dose-response behavior was identical to NAADP alone. This is shown in Fig. 8D, where the red squares indicate the dose-response curve resulting from ADPR supplementation of NAADP at various concentrations (n = 5-9). The first EC₅₀ (EC₅₀₁) of the dose-response fit to the ADPR plus NAADP data extracted at 100 s whole-cell time was calculated to be 1.1 µM with a Hill coefficient of 3, whereas the second component, EC₅₀₂, was 116 µM with a Hill coefficient of 2. Fig. 8C depicts example I/V curves taken at 100 s from a cell perfused with 3 µM NAADP only, which failed to activate any currents (blue trace), and a cell co-perfused with 3 µM NAADP in the presence of 100 nM ADPR (red trace). These data confirm that TRPM2 can be activated in a primary cell system expressing TRPM2 channels in the plasma membrane. In addition, with an EC₅₀ of 95 µM, NAADP is about 8-fold more effective in recruiting TRPM2 currents in neutrophils than it is in the overexpression system (EC₅₀ of 730 µM) and possibly Jurkat T cells⁶⁴.
**Figure 8: ADPR and NAADP synergize to activate TRPM2 currents.** (A) Average normalized TRPM2 currents activated by NAADP in human neutrophils (n = 3-5). Intracellular conditions and data acquisition/analysis were as in Fig. 1A. (B) Average normalized TRPM2 currents activated by various NAADP concentrations in the presence of 100 nM ADPR (n = 5-9). Intracellular conditions and data acquisition/analysis as in (A). (C) I/V curves taken from representative cells perfused with 1 mM NAADP (black trace), 3 µM NAADP (blue trace) or 3 µM NAADP + 100 nM ADPR (red trace). (D) Dose-response curves of TRPM2 currents evoked by NAADP (black circles, n = 3-5) or subthreshold ADPR (100 nM) + increasing NAADP concentrations (red squares, n = 5-9) in unbuffered K⁺-based internal solution. Current amplitudes were plotted against concentrations and fitted with dose-response curves. The EC₅₀ values are indicated in the panel. Hill coefficient was 1.6 for NAADP. A two-component dose-response curve was fitted to the NAADP data supplemented with 100 nM fixed ADPR. Here, the Hill coefficients were 3 for EC₅₀₁ and 2 for EC₅₀₂.

**TRPM2 in mouse neutrophils**

Experiments were further carried out using neutrophils isolated from spleen of C57/BL6 mice. Activation of TRPM2 currents by ADPR was characterized and the functional knock-down was confirmed by the use of newly available TRPM2 knock-out mice provided by the group of Yasuo Mori (Kyoto, Japan). In addition, to assess the functional role of endogenously generated ADPR, knock-out mice lacking the ectoenzyme CD38 were used. A possible involvement of CD38-synthesized ADPR as TRPM2 agonist had been proposed previously. Because CD38’s main catalytic product may determine channel function, it was assessed whether its expression in neutrophils had any influence on TRPM2 channel activity. Therefore, experiments were conducted using CD38-deficient mice.
Regulation of TRPM2 by ADPR in wild-type, TRPM2 and CD38 deficient mouse neutrophils

ADPR dose-response curves of neutrophils isolated from mouse spleen of wild-type and cd38 knock-out mice were established. ADPR-evoked currents, measured at –80 mV and 100 seconds into the experiment, were extracted, averaged, normalized to cell size and plotted versus their respective ADPR concentration (Fig. 9A; black circles, n = 3-6 and cd38 knock-out red circles, n = 3-10). A dose-response fit to the data yielded a half-maximal effective concentration (EC$_{50}$) for ADPR of 600 nM for TRPM2 wild-type cells and 500 nM for the CD38 knock-out cells both with a Hill coefficient of 2. Figure 9B shows the average normalized time course of inward TRPM2 current development measured in wild-type and cd38$^{-/-}$ neutrophils assessed at –80 mV. Currents were evoked by 10 µM intracellular ADPR added to the standard K$^+$-glutamate-based pipette solution. Following the same protocol for neutrophils isolated from trpm2$^{-/-}$ mice, no inward currents could be seen even when using concentrations of 1 mM ADPR in the standard internal solution. Note here that trpm2$^{-/-}$ neutrophils were isolated from mouse bone marrow. In all cases, no exogenous Ca$^{2+}$ chelators were used to adjust intracellular calcium concentration ([Ca$^{2+}$]$_i$). In case of wild-type and cd38$^{-/-}$, the evoked currents showed typical characteristics of TRPM2 with a linear current-voltage (IV) relationship and a reversal potential ($E_{rev}$) of 0 mV (Fig. 9C). These currents were completely absent in the trpm2$^{-/-}$ mouse. Figure 9D shows staining (provided by Adriana Sumaoza-Toledo from the laboratory of Santiago Partida-Sanchez, Columbus Ohio) of bone marrow-derived wild-type neutrophils using rabbit anti mouse antibody targeting TRPM2 (see materials). Cells were co-stained with DAPI to indicate nuclei.
Figure 9: Activation of TRPM2 currents by ADPR in Wt, cd38−/− and trpm2−/− mouse neutrophils. (A) Dose-response curves of ADPR-induced TRPM2 currents in unbuffered internal solution of wild-type neutrophils (black circles, n = 3-6) and cd38 knock-out (red circles, n = 3-10). Data were plotted against ADPR concentrations and fitted with dose-response curves. The EC50 values are indicated in the panel. Hill coefficients were 2 for wild-type and CD38 knock-out. To establish the dose-response curves, inward currents at −80 mV were extracted at 100 sec, averaged and plotted versus the respective ADPR concentration. (B) Average normalized TRPM2 currents activated by ADPR in mouse neutrophils. Currents were measured with a voltage ramp from −100 mV to +100 mV over 50 ms at 0.5 Hz intervals from a holding potential of 0 mV. Inward current amplitudes were extracted at −80 mV, averaged and plotted versus time. Cells were perfused with the standard intracellular K+-based solution in the absence of exogenous Ca2+ buffers and supplemented with concentrations of 10 µM ADPR in wild-type and CD38 knock-out mouse neutrophils and 1 mM ADPR in TRPM2 knock-out cells as indicated (n = 5-10). Standard extracellular solution contained 1 mM Ca2+. (C) Current-voltage (I/V) curves taken from representative cells perfused with 10 µM ADPR in wild-type, CD38 knock-out and 1 mM ADPR in TRPM2 knock-out mice. (D) Immunostaining of mouse neutrophils treated with polyclonal rabbit anti human TRPM2 antibody (right picture, green). Nuclei are labeled by DAPI (left picture, blue). The staining was greatly provided by Dr. Adriana Sumoza-Toledo.

**TRPM2 and calcium-influx channels in monocytes**

*Wild-type mouse monocytes express ADPR-sensitive currents that are absent in monocytes isolated from TRPM2 knock-out mice.*

The laboratory of Yasuo Mori from the Department of Synthetic Chemistry and Biological Chemistry, Kyoto University in Japan, recently designed a TRPM2 knock-out mouse. While Dr. Mori’s laboratory conducted the biochemical assessment of signaling pathways involving TRPM2, the electrophysiological investigation was performed in our laboratory. It had been known that the human monocytic cell line U937104 highly expresses functional TRPM221. Based on Dr. Mori’s findings of TRPM2 involvement in
inflammatory processes in monocytes, the activation of the channel by ADPR was investigated in wild-type and trpm2<sup>−/−</sup> monocytes. First, a dose-response curve of ADPR-induced currents was established in monocytes isolated from wild-type mice (C57BL/6) peripheral blood (see figure 10A). To this end, cells were perfused with standard internal solution supplemented with increasing concentrations of ADPR in the absence of intracellular calcium chelators. K-glutamate was substituted with Cs-glutamate in order to silence activation of any potassium channels contaminating the TRPM2-related current. Data were acquired by applying the standard voltage-ramp protocol ranging from −100 mV to +100 mV and of 50 ms duration. Current amplitudes were measured at −80 mV and 100 s into the experiment, normalized for cell size, averaged and plotted against the respective ADPR concentration (<i>n</i> = 5-7 ± S.E.M.) (see Figure 10A). A fit to the data points yielded an <i>EC<sub>50</sub></i> = 25 µM with a Hill coefficient of 1. Figure 10B shows a time course of current development at −80 mV induced by perfusion of monocytes isolated from wild-type mice (closed circles, <i>n</i> = 6) or trpm2<sup>−/−</sup> mice (open circles, <i>n</i> = 11) with 1 mM ADPR. Peak currents were around 400 pA/pF. No currents were triggered by ADPR in the TRPM2 knock-out cells. Figure 10C shows the current-voltage (I/V) relationship of data traces extracted from representative cells 100 s into the experiment, isolated from wild-type (wt) or trpm2<sup>−/−</sup> mice (KO). Cells were perfused with 1 mM ADPR. Data were not leak-subtracted to exemplify the background current unrelated to ADPR-induced currents. Note the absence of any currents characteristic of TRPM2.
**Figure 10:** Activation of TRPM2 currents by ADPR in Wt and trpm2<sup>−/−</sup> mouse monocytes. (A) Dose-response curves of ADPR-induced TRPM2 currents in unbuffered internal solution of wild-type monocytes (black circles, n = 5-7). Data were plotted against ADPR concentrations and fitted with dose-response curves. The EC<sub>50</sub> value was 25 µM with a Hill coefficient of 1. To establish the dose-response curves, inward currents at −80 mV were extracted and taken at 100 sec, averaged and plotted versus the respective ADPR concentration. (B) Average normalized TRPM2 currents activated by 1 mM ADPR. Currents were measured with a voltage ramp from −100 mV to +100 mV over 50 ms at 0.5 Hz intervals from a holding potential of 0 mV. Inward current amplitudes were extracted at −80 mV, averaged and plotted versus time. Cells were perfused with the standard intracellular Cs<sup>+</sup>-based solution in the absence of exogenous Ca<sup>2+</sup> buffers and supplemented with concentrations of 1 mM ADPR in wild-type and TRPM2 knock-out mouse monocytes as indicated (n = 5-10). Standard extracellular solution contained 1 mM Ca<sup>2+</sup>. (C) Current-voltage (I/V) curves taken from representative cells at 100 seconds perfused with 1 mM ADPR in wild-type and TRPM2 knock-out mice.

**H<sub>2</sub>O<sub>2</sub>-induced TRPM2, I<sub>CRAC</sub> and TRPM7 in wild-type and trpm2<sup>−/−</sup>**

Recruitment of monocytes in inflammatory processes depends on chemokine-signaling.<sup>105</sup> It had been shown previously that reactive oxygen species (ROS) contribute to initiation of the localization process of monocytes to inflammatory sites. In addition, ROS is a well-known activator of TRPM2. The collaborative work with Dr. Mori’s laboratory Yamamoto et al.<sup>106</sup> demonstrate that ROS-induced chemokine production in monocytes is controlled by the activation of TRPM2.

To test whether perfusion of ROS alone would lead to activation of TRPM2-mediated currents, experiments were carried out perfusing monocytes with H<sub>2</sub>O<sub>2</sub>. In addition, it was investigated whether other calcium-conducting channels were affected by the knock-out of TRPM2, which then might alter calcium-dependent production of chemokines. Therefore, the newly identified and well characterized CRAC (calcium release-activated calcium current) channel (Orai1/CRACM1)<sup>29, 32</sup> and TRPM7<sup>107</sup> were investigated by comparing wild-type and knock-out mice. In the first set of experiments, wild-type and TRPM2 knock-out monocytes were perfused with standard Cs-based internal solution supplemented with 100 µM hydrogen peroxide. Data were acquired by applying a voltage-ramp protocol ranging from −100 mV to +100 mV and of 50 ms duration. Figure
11A shows the time-course of TRPM2 current development at –80 mV induced by perfusion of monocytes isolated from wild-type mice (open circles, n = 8) or trpm2−/− mice (closed circles, n = 11) with 100 μM H2O2. Maximum peak currents were around 60 pA/pF, which is about eight times lower than can be achieved with saturating concentrations of ADPR. No currents were triggered in the TRPM2 knock-out cells. Figure 11 B shows the current-voltage (I/V) relationship of data traces extracted from representative cells 300 s into the experiment and either isolated from wild-type (wt) or trpm2−/− mice (KO). Cells were perfused with 100 μM H2O2. Note the absence of any currents characteristic of TRPM2.

In a separate set of experiments, CRAC channels were assessed. Here, cells were perfused with Cs-based standard internal solution supplemented with 20 μM inositol 1,4,5 trisphosphate and Cs-BAPTA to induce store depletion, triggering subsequent I_{CRAC} activation. Standard external solution was used, except that it contained higher concentrations of 10 mM calcium. Data were acquired by applying a voltage-ramp protocol every 2 seconds ranging from –150 mV to +150 mV and of 50 ms duration. Development of I_{CRAC} showed similar kinetics in wild-type and trpm2−/− with a peak of 0.7 pA/pF of inward current at –80 mV (Figure 11C). Note here that leak current was corrected by subtraction of the 2nd or 3rd ramp recorded after whole-cell break-in from all subsequent ramps recorded. Figure 11D shows an example current voltage relationship of I_{CRAC} with a reversal potential of about 50 mV, exhibiting similar characteristics for wild-type and trpm2−/− cells. Another calcium-conducting pathway in monocytes is TRPM7 (transient receptor potential melastatin 7), which is regulated by multiple factors like Mg^{2+} ions, energy levels (ATP) and phosphoinositides^{108}. To measure TRPM7, the same ramp protocol was used as for TRPM2. Internal solution was Cs-based and in addition supplemented with 10 mM BAPTA and 700 μM free Mg^{2+}. Figure 11E shows the average current development of MagNuM (magnesium nucleotide-regulated metal; TRPM7) in wild-type (open circles, n = 5) and trpm2−/− monocytes (closed circles, n = 5). Current amplitudes were measured at –80 mV and +80 mV, normalized to cell size, averaged and plotted versus time of the experiment. Data were not leak subtracted. Figure 11D displays example current-voltage relationships of MagNuM currents measured in wild-type (black trace) or trpm2−/− monocytes (red trace), which are characteristic for
TRPM7 by exhibiting a large outwardly rectifying current. I/V data are leak corrected by subtracting the 3rd ramp. To estimate whether cell size was affected by the TRPM2 knock-out, the distribution of cell size (in capacitance; pF) from wild-type and trpm2−/− mouse was compared (Figure 11G). The average cell size of wt cells was 8 +/- 0.5 pF (n = 79) and for trpm2−/− cells was 5.7 +/- 0.7 pF (n = 73). Figure 11H correlates $I_{\text{CRAC}}$ current density (pA/pF) to respective cell size. Currents were assessed at -80 mV and 150 s into the experiment. Note that wild-type and KO cells overlap, except for three cells that were small (around 2.2 pF) but had larger CRAC currents than all other cells (around -1.5 pA/pF), which might be due to contamination by other blood cells through the isolation process.

Figure 11: H$_2$O$_2$-induced TRPM2, CRAC and MagNuM currents in monocytes isolated from wild-type or TRPM2 KO mice. (A) Average current development induced by 100 µM H$_2$O$_2$ in the standard Cs-based pipette solution and measured in wild-type monocytes (open circles, n = 8) or monocytes isolated from TRPM2 KO mice (closed circles, n = 11). Data were acquired using a voltage ramp from -100 mV to +100 mV over 50 ms at a rate of 0.5 Hz. Current amplitudes were measured at -80 mV, normalized to cell size, averaged and plotted versus time of the experiment. Data were corrected by subtracting break-in leak currents. Error bars indicate S.E.M. (B) Current-voltage relationship of TRPM2 evoked by intracellular
H$_2$O$_2$ (100 µM) and extracted from an example wild-type cell (black trace) or example TRPM2 KO cell (red trace) at 300 s. (C) Average time course of $I_{CRAC}$ development measured in wild-type (open circles, n = 11) or TRPM2 KO monocytes (closed circles, n = 18). Data were analyzed as in (A). The external solution contained (in mM): 10 CaCl$_2$, 140 NaCl, 2.8 KCl, 2 MgCl$_2$, 10 HEPES-NaOH. The internal solution contained (in mM): 120 Cs-glutamate, 3 MgCl$_2$, 8 NaCl, 10 HEPES-CsOH, 10 CsBapta, 0.02 inositol 1,4,5 trisphosphate. Data were leak-corrected by subtraction of the 2$^\text{nd}$ or 3$^\text{rd}$ ramp recorded after whole-cell break-in from all ramps recorded per cell. (D) Average current-voltage curves of CRAC currents measured in wild-type cells (black trace, n = 3) or TRPM2 KO cells (red trace, n = 5). (E) Average current development of MagNuM (TRPM7) in wild-type (open circles, n = 5) and TRPM2 KO monocytes (closed circles, n = 5). Current amplitudes were measured at −80 mV and +80 mV, normalized to cell size, averaged and plotted versus time of the experiment. Data were not leak subtracted. (F) Current-voltage relationship of MagNuM currents measured in example wild-type (black trace) or TRPM2 KO monocytes (red trace). IV data are leak corrected by subtracting the 3$^\text{rd}$ ramp. (G) The graph correlates $I_{CRAC}$ current density (pA/pF) measured in individual wild-type (black dots) or TRPM2 KO monocytes (red dots) to their respective cell size. Currents were assessed at 150 s into the experiment. (H) The graph plots $I_{CRAC}$ amplitude (in pA) measured in individual wild-type (black dots) or TRPM2 KO monocytes (red dots) against their respective cell size. Same data set as in (G), but not normalized for cell size.

**Effects of intracellular AMP on receptor-mediated calcium release**

*Adenosine-mono-phosphate inhibits IP$_3$ receptor-mediated calcium release*

While elucidating the role of ADPR and TRPM2 as a calcium release channel, it arose that AMP had an additional effect apart from antagonizing ADPR-induced TRPM2 currents across either ER or plasma membrane. It turned out that AMP also seemed to have an effect on G-protein-coupled pathways. While acting on TRPM2 in a weak manner by shifting the EC$_{50}$ to higher concentrations of ADPR, concentrations of AMP in the lower µmolar range strongly inhibited the calcium release provoked by receptor-stimulated pathways. In order to reveal the ubiquitous nature of AMP to antagonize different receptor-mediated calcium release, experiments were performed using different agonists, targeting different receptor classes, as well as using cell lines or primary cells derived from different species. First it was assured that triggering different G-protein-coupled receptors resulted in IP$_3$-induced calcium release. Application of 100 µM ATP, presumably acting on P2Y receptors$^{84}$ in wild-type HEK293 cells, caused a transient release of calcium from stores under patch-clamp whole-cell conditions (figure 12A). Data were obtained using external solutions deficient of calcium, using internal K-based standard solution that included 200 µM Fura-2 and different supplements were added as indicated. To confirm that this signal was mediated by the production of IP$_3$, cells were perfused with 100 µg/ml heparin, which blocks IP$_3$ and inhibits calcium transients. In a similar manner, 100 µM AMP antagonized this signal. Next the PAR (protease-activated receptor) – receptor agonist thrombin was used$^{109}$. While 20 U/ml of thrombin

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functionally provoked a distinct transient calcium release, it was clearly antagonized by 100 μM AMP (Fig. 12B). This was also the case both for the muscarinic receptor agonist carbachol and the previously investigated P2Y agonist ADPR (see Fig. 1A). In both sets of experiments, the presence of 100 μM internal AMP entirely inhibited the stimulated calcium-release pathway (Figure 12C and D). Next, this mechanism was investigated in isolated mouse primary pancreatic beta cells. To this end, the same protocol was applied as described for HEK293 (see above). Internal solution was Cs-based and cells were held at a holding potential of −70 mV. Figure 12E displays the action of 100 μM carbachol, which is antagonized by the IP₃ receptor inhibitor heparin (100 μg/ml). In analogy to the response in HEK293 cells, this transient was also blocked by the use of 100 μM AMP (Figure 12E). It had been shown previously, that in mouse primary beta cells only P2Y receptor family is involved in mediating ADPR-induced signaling pathways (see Figure 4E). As illustrated in Fig. 12F, 100 μg/ml heparin completely suppressed the response, indicating that it was entirely mediated by IP₃. Likewise AMP alone was able to act as an inhibitor of the P2Y receptor-mediated calcium release. As another primary cell type employing different G-protein-coupled receptors, experiments were carried out in mouse bone marrow-derived immature dendritic cells. Mouse dendritic cells express functional leukotriene B4 (BLT1/2 receptors) receptor, whose stimulation leads to elevated calcium levels, necessary for dendritic cell migration. Fig. 12G shows, that the effect of stimulation with 500 nM LTB₄ in calcium-deficient external standard solution induced calcium release (control, standard internal K-based solution) that is entirely mediated by the production of IP₃. Here, low concentrations of 10 μM AMP suppressed this chemokine-mediated signal.
External ADPR mediates IP$_3$-independent calcium release in INS-1 cells

By extending the investigations on the effect of AMP on G-protein-stimulated pathways it was found that in INS-1 (rat pancreatic beta cell line) cells there seemed to be a second pathway downstream of receptor stimulation that did not depend on IP$_3$-production. The
“classical” model for IP₃ production through muscarinic receptors showed the same characteristics as observed in the HEK293 and primary mouse pancreatic beta cells. Thus, INS-1 cells responded to application of 100 µM carbachol as expected for the IP₃ pathway and could be inhibited at different points in the signaling pathway. On the level of G-protein, 500 µM GDP-β-S abolished the response. Blocking phospholipase C (PLC) by 10 µM U73122 and the IP₃–receptor by heparin also abolished the response (see Fig. 13a). Interestingly, AMP also seemed to inhibit this pathway in a concentration dependent manner, even showing an effect at concentrations as low as 1 µM of internal AMP, completely blocking Ca²⁺ release at 10 µM (Fig. 13B). Experimental conditions in Fig. 13 were similar to Fig. 12, with a K-based internal solution containing 200 µM Fura-2 and using different pharmacological tools on the external and internal side. Cells were held in the whole-cell configuration at – 70 mV. Surprisingly, the calcium release in response to external 30 µM ADPR could neither be blocked by 500 µM GDP-β-S nor by heparin (100 µg/ml) (see Fig. 13C), although the use of 10 µM U73122 abolished this transient, and instead caused a slow rise of calcium, followed by a delayed loss of membrane integrity. Next, it was investigated whether another calcium release channel might be involved (Fig. 13D) in ADPR signaling. Cells were co-perfused with high concentrations of ryanodine (40 µM) that inhibit the ryanodine receptor (RyR) and 100 µg/ml heparin. This combination did not block ADPR-mediated release. Also 100 µM of AMP alone was not sufficient to abolish this signal. Only co-perfusion of both heparin and AMP was able to antagonize the effect of ADPR, indicating their different nature of action. Heparin would be acting on the IP₃ receptor, while the target of AMP remains to be elucidated. In order to find out, whether this novel AMP-sensitive pathway was either mediated through the P2Y receptor family or the adenosine receptor family, both receptor types were pharmacologically silenced while inhibiting the IP₃ receptor through internal heparin. Fig. 13E shows the response triggered by 100 µM external ADPR in the presence of internal heparin and the unspecific adenosine receptor antagonist CGS-15943 in the bath. The presence of a calcium release indicates that a heparin-insensitive factor downstream of P2Y receptors might account for calcium release. In fact, under experimental condition where both the IP₃ receptor through heparin and the P2Y receptor family through suramin were inhibited, no signal could be mediated by ADPR. This
clearly demonstrated that the AMP-sensitive pathway was mediated by suramin-sensitive receptors, likely P2Y receptors, coupling to a novel release channel. As an approach to identify the release channel, the TRPV1 channel was considered as a possibility, as it is known to be highly expressed in pancreatic beta cells. Interestingly, the TRPV1 antagonist capsazepine, in combination with heparin, could entirely suppress the ADPR-mediated response at a concentration of 20 µM (Fig. 13F). Capsazepine alone, though, was not sufficient to block the response. Fig. 13G demonstrates pharmacologically the involvement of different adenosine receptor subtypes. Cells were incubated with 100 µM suramin to antagonize the P2Y receptors. Cells were loaded with Fura-2-AM and 100 µM ADPR was applied, with inhibitors continuously present in the bath. The adenosine receptor 1 inhibitor (A1) DPCPX (1 µM) in combination with suramin failed to suppress the transient. It even failed to suppress the signal together with the A2A- (1 µM SCH 58261) or A2B- inhibitor (1 µM MRS 1754). Only the use of all three specific adenosine receptor inhibitors, including suramin, silenced the ADPR-mediated signal, indicating the involvement of at least three different subtypes of adenosine receptor in this model.
Figure 13: AMP inhibits muscarinic receptor-induced calcium-release pathway in rat INS-1, but requires heparin in addition to ADPR-induced calcium release. (A) The graph displays Fura-2 experiments. Averaged Ca^{2+} signal in whole cell patch-clamped HEK293 cells with Fura-2. Whole-cell break-in was before application start (not shown). Application start of 100 µM Carbachol in the absence of extracellular Ca^{2+} as indicated by the arrow. The internal Cs-based solution contained 200 µM Fura-2 for the control (black trace, n = 5) and 500 µM GDP-β-S (blue trace n = 6) or 100 µg/ml heparin (red trace, n = 6). The red trace represents data where the cells were perfused with internal solution supplemented with 200 µM Fura-2 and 10 µM U73122 in the bath (gray trace n = 5). (B) Same protocol as in A, but dose response for inhibitory effect of AMP (1 µM n = 11, 10 µM n = 7, control n = 5). (C) Same as A, but with extracellular 30 µM ADPR. Neither 500 µM GDP-β-S (black trace n = 7) nor 100 µg/ml heparin (red trace, n = 6) inhibit the effect of ADPR. 10 µM U73122 (red trace n = 5) inhibit effect of ADPR under conditions as in A. In figure D neither the effect of 30 µM ADPR can be antagonized by 100 µM AMP alone (black trace n = 7) or by 100 µg/ml heparin in combination with 40 µM ryanodine (blue trace n = 6). Only heparin in addition with AMP antagonizes this pathway (red trace n = 7). Figure E displays application of 100 µM ADPR triggering calcium release in the presence of 1 µM CGS 15943 in the bath solution and 100 µg/ml heparin in the patch pipette (black trace n = 5). Transient is inhibited by the use of same concentration of intracellular heparin and omission of 100 µM suramin to the bath solution (red trace n = 5). Figure F displays that the effect of intracellular AMP on extracellular ADPR can be substituted by capsazepine. 20 µM Capsazepine alone does not inhibit the calcium transient induced by 100 µM ADPR (black trace n = 7), but antagonizes in combination with 100 µg/ml heparin (red trace n = 6). Figure G elucidates involvement of adenosine receptors. Cells challenged with 100 µM ADPR extracellularly, inhibited with 100 µM suramin and addition of 1 µM DPCPX in bath (black trace n = 9). Blue trace same but DPCPX in addition with either SCH 58261 1µM (n = 7) or MRS 1754 (grey trace n = 9). Only the use of all three inhibitors abolishes the signal caused by external ADPR supplemented with internal heparin (red trace n = 6). All experiments under figure 13 are patched in the whole cell configuration under same conditions of internal standard Cs-based solutions containing 200 µM Fura-2 supplemented with pharmacological tools as indicated. External solution was standard with supplements as indicated individually.
DISCUSSION

Nucleotide signaling in the model of HEK293 cells and Pancreatic beta cells

TRPM2 is a relatively novel nonselective cation channel conducting mono- and divalent ions, with the rare ability of performing dual functions, with channel properties as well as pyrophosphatase activity mediated through its NUDT9-h enzymatic domain. Relatively little is known about its physiological role, or what biochemical pathways it mediates. Since it conducts calcium, it evidently has the potential to participate in the modulation and/or amplification of calcium signals. This study aimed at understanding the role of TRPM2 and its agonist ADPR in calcium signaling. As ADPR had been reported to act on the extracellular side through G-protein-coupled receptors to mediate calcium signals, this study aimed at differentiating the aspects of ADPR functions on the mobilization of calcium as a first and second messenger and the novel function of TRPM2 acting as a calcium release channel in intracellular stores.

The individual calcium signaling events were first investigated in the HEK293 cell line. It could be shown that stimulation with external ADP-ribose triggers a pathway, that involves P2Y receptors, G-proteins, activation of PLC, production of IP₃, and finally the gating the of the IP₃ receptor. The possible ADPR metabolites, cADPR and β-NAD, were not involved in this receptor-stimulated pathway. Once the external action of ADPR was understood, key experiments were performed by selectively inhibiting receptor-stimulated pathways using pharmacological tools. Experiments were performed perfusing cells intracellularly with ADPR while inhibiting G-protein-coupled pathways. Here it could be demonstrated that ADPR caused calcium release in the HEK293 system overexpressing TRPM2, but not in wild-type cells that lack the channel. This finding provided evidence that TRPM2 potentially was able to act as a calcium-release channel in addition to its plasma membrane functions.

TRPM2 channels are found in various kinds of excitable and non-excitable cells, including neuronal tissues and immune cells. In order to investigate whether TRPM2 could play a role in releasing calcium in physiological relevant models, experiments were expanded to include an electrically excitable cell type model, by using the monoclonal rat pancreatic beta cell line INS-1. These cells are known to express functional endogenous TRPM2 and possess calcium stores. Furthermore, a possible function of TRPM2 in these
cells might be of relevance in the regulation of insulin release, a mechanism that is known to be regulated by intracellular calcium levels\textsuperscript{114-116}. The efficacy of ADPR to gate TRPM2 channels in this insulin-secreting model system was briefly characterized. ADPR activated TRPM2 with an EC\textsubscript{50} of around 100 µM and its action antagonized by 10-fold higher concentrations of adenosine mono phosphate (AMP)\textsuperscript{63}. Interestingly, this is one order of magnitude higher than described in the HEK293 overexpressing TRPM2 (EC\textsubscript{50}~12 µM)\textsuperscript{21, 63} and even two orders higher than observed in primary human neutrophils (EC\textsubscript{50}~1 µM). Still, the relationship of endogenous ADPR measured through HPLC by two different groups\textsuperscript{86, 117} does not seem to correlate well with the levels of TRPM2 activation under experimental conditions. However this discrepancy might be explained by the fact that TRPM2 can be regulated via multiple modulators. For example elevated levels of calcium strongly up-regulate channel activity\textsuperscript{98} in the presence of agonist. Conversely, chelation of this ion conditions completely abolishes channel activation in native cells (personal observation). An additional mechanism that affects channel activity is the calcium-binding protein calmodulin, which can up-regulate the channel by acting on calmodulin-binding sites within the channel\textsuperscript{59}. It had been reported that phosphorylation of TRPM2 also contributes to channel regulation. The widely expressed tyrosine phosphatase PTPL1 negatively regulates channel activity by decreasing tyrosine phosphorylation of TRPM2\textsuperscript{118}. All these modulators contribute to the overall gating of TRPM2, in addition to its main agonist ADPR. Experiments following the same strategy as in HEK293 to resolve the effects of extracellular ADPR on receptor-stimulated pathways in INS-1 cells, implicated another possible cell-surface receptor that may relay ADPR signaling through G-proteins, namely nucleotide receptors. Here it was only possible to inhibit the ADPR-induced signal using a combination of P2Y and Adenosine receptor blockers. Overall, the potency of ADPR in causing receptor-mediated calcium release in INS-1 cells was 1-2 orders of magnitude higher than in HEK293 cells or primary mouse pancreatic beta cells. This was not due to the additional presence adenosine receptors, as the unspecific inhibitor suramin was considerably more effective than the adenosine inhibitor CGS-15943 in suppressing the response to low concentrations (10 µM) of ADPR. This suggests that P2Y receptors
possess higher sensitivity to ADPR in INS-1 cells than adenosine receptors. This higher efficacy might be due to different expression patterns of P2Y receptor subtypes in INS-1 cells.

Since the ADPR response in HEK293 cells is also mediated through P2Y receptors, it would appear that either species differences or the P2Y receptor subtype-complements of rat INS-1 vs. human HEK293 cells account for the differences in ADPR sensitivity. HEK293 cells primarily express P2Y subtypes 1, 2, and 4, although a slightly differing P2Y receptor complement has been reported for these cells as well. INS-1 cells express subtypes 1, 2, 4, 6, and 12, which are expressed at similar levels. Thus it would seem that a specific P2Y receptor subtype-complement might determine the high-affinity response to ADPR in INS-1 cells, although it cannot entirely be ruled out that species differences, isoforms or clonal variation might play a role as well. A more extensive pharmacological profiling of P2Y receptors in INS-1 cells should be able to resolve this question.

The ADPR precursors NAD+ and cADPR were tested for efficacy in evoking Ca2+ release responses in INS-1 cells. In marked contrast to HEK293 cells, where these molecules failed to induce Ca2+ release, both NAD+ and cADPR were able to trigger Ca2+ release transients in INS-1 cells, although cADPR did so more efficiently than NAD+. From functional studies in HEK293 cells, the maximal levels of contamination of these compounds with ADPR were determined to be ~1-3%. Since the threshold concentration for these compounds is 10 to 30-fold higher than that of ADPR, it indicated that the NAD+- and cADPR-mediated Ca2+ release activity is clearly not caused by nucleotide contamination. It rather might be either due to a genuine agonistic action of these compounds on cell surface receptors or caused by exogenous metabolic conversion to ADPR. The ectoenzyme CD38 which converts NAD and cADPR into ADPR is expressed at high levels in beta cells, but presumably less so in HEK293 cells. The NAD+- and cADPR-mediated responses might as well be caused by conversion of these molecules to ADPR. This hypothesis is supported by the fact, that the cADPR response is almost completely antagonized by the analog 8-Br-ADPR, which possibly competes with metabolized ADPR for receptor binding. Further studies in mutant mice may strengthen this hypothesis. Both NAD+ and cADPR become ineffective in causing Ca2+ release in
primary beta cells of transgenic mice deficient in CD38 expression. Consistent with this idea is the fact that, like ADPR, both NAD\(^+\)- and cADPR effects are mediated through P2Y and adenosine receptors, since the combined suppression of these receptors by suramin and CGS-15943 completely antagonizes the response. Furthermore, ADPR and cADPR show a similar pharmacological profile, since suramin also is more effective than CGS-15943 in suppressing the response to cADPR.

Next, it was considered that TRPM2 could also function as a release channel in INS-1 cells. The presence of TRPM2 was confirmed through immunocytochemical experiments and showed both a peripheral and intracellular location of the protein. Interestingly, TRPM2 rarely co-localized with ER markers. Instead TRPM2 fluorescence appeared as punctate stains distributed throughout the cell, possibly reflecting localization in vesicular compartments. Future immunocytochemical experiments, using specific intracellular markers for various organelles, like lysosomes, mitochondria or insulin-secreting vesicles, will likely reveal the precise subcellular localization of TRPM2 channels.

The physiological assessment of intracellular action of ADPR demonstrated that the agonist elicited a sizeable increase in [Ca\(^{2+}\)]\(_i\), in a concentration dependent manner, while P2Y and adenosine receptors where pharmacologically silenced. Interestingly, this could also be achieved by perfusion of reactive oxygen species (H\(_2\)O\(_2\)) in the presence of IP\(_3\)-receptor inhibitors. This is consistent with ROS-mediated ADPR production through the PARP/PARG pathway, resulting in the activation of TRPM2 in the plasma membrane\(^6\). It was further determined that the commonly known calcium release channels like the IP\(_3\) receptor or the ryanodine receptor, both of which are expressed in pancreatic cells\(^{119,120}\), could not account for the ADPR-induced calcium release, since neither heparin nor the plant alkaloid ryanodine (>20 \(\mu\)M) significantly affected the ability of ADPR to mobilize calcium from stores\(^{121}\). The rather small effect of ryanodine might be due to the complex nature of the ryanodine receptor being regulated through calcium,\(^{122}\) as well as having both low- and high-affinity binding sites for its agonist\(^{123}\). It is also possible that TRPM2 and ryanodine receptors may be co-localized partially in the same stores. Whatever the nature of the store might be and the release channels it expresses, pharmacological tools revealed that intracellular TRPM2 resided in stores that express SERCA, as depletion with the sarco/endoplasmic reticulum calcium ATPase (SERCA) inhibitor thasigargin\(^{124}\)
prevented ADPR from releasing calcium. Importantly, the ADPR-mediated calcium mobilization could directly be linked to TRPM2 expression, as calcium release was essentially eliminated by knockdown of TRPM2 using TRPM2-specific siRNA, but not a scrambled control siRNA. The efficacy of specific siRNA knockdown of TRPM2 was confirmed by the TRPM2 reduction of channel activity in the plasma membrane, whereas scrambled siRNA had no effect. Together, these data establish that TRPM2 proteins in INS-1 beta cells, as in the heterologous overexpression system, function as both Ca\(^{2+}\)-permeable cation channels in the plasma membrane and as Ca\(^{2+}\) release channels in intracellular stores.

Although the INS-1 cell line is considered an excellent model to study beta-cell physiology, as it is of monoclonal origin and possesses all of the crucial mechanisms required for insulin secretion, it might not fully reflect the properties of primary cells. Also species-specific variations might account for different physiological properties. Therefore, the study was extended to primary beta cells of C57BL/6 mice and the results from these experiments were comparable to those obtained in the rat insulin secreting cell line INS-1. Maximum inward currents were similar, although the EC\(_{50}\) for ADPR was roughly 3-fold higher than in the cell line. This reduced potency was also apparent when analyzing the efficacy of ADPR to trigger calcium release in primary cells, where a slightly higher concentration was necessary compared to INS-1 cells.

One can only speculate about the reasons for the slight differences observed. One explanation would be, that expression profile of proteins might change after transformation to a cell line. This has been observed for example for T-lymphocytes. Jurkat T-cells respond to ADPR with an EC\(_{50}\) of 7 µM\(^{64}\), whereas T-cells isolated from human peripheral blood completely lack any TRPM2-like currents (personal observation). In addition, differentiation-states of individual cells from freshly isolated islets might contribute to this discrepancy.

Inhibition of the ADPR-induced currents by AMP was similar as in every cell system investigated, although in primary cells, a significantly higher concentration of AMP than ADPR was necessary to suppress TRPM2 currents. The relatively weaker ability of AMP to antagonize ADPR-induced currents might be due to the fact, that the NUDT9 homology region (NUDT9-H) at the C-terminus of the channel, which acts as an ADPR
pyrophosphatase, exhibits lower enzymatic activity than the NUDT9 enzyme expressed in mitochondria, resulting in a lower affinity of AMP$^{21, 58}$. Furthermore, additional regulatory factors might counterbalance the antagonistic effect of AMP in these cells.

To assure that ADPR-induced currents with their characteristic current-voltage relationship were entirely conferred by TRPM2, beta cells were isolated from a TRPM2 knock-out mouse, provided by the group of Yasuo Mori$^{80}$. Perfusion of these beta cells with high concentrations of ADPR did not provoke a current specific for TRPM2.

The action of extracellularly applied ADPR surprisingly showed that, in contrast to the rat INS-1 cell line, only P2Y receptors were involved in the calcium-release response, similar to the situation in HEK293 cells. Also, the efficiency of ADPR to trigger the release was similar compared to HEK293, being 30-fold higher than in the insulinoma cell line. Whether this observation is due to different isoforms of P2Y receptors or the lack of functional adenosine receptors in primary mouse cells remains to be determined and was not further investigated. Stimulation with extracellular cADPR mobilized store calcium in a manner similar to INS-1, albeit at higher threshold concentrations compared to the ADPR stimulus. This supports the hypothesis that cADPR is hydrolysed to ADPR by the ectoenzyme CD38$^{81, 96}$, subsequently acting on P2Y receptors. Surprisingly, β-NAD failed to elicit calcium signals even at high concentrations. At first it was speculated that a two-step reaction, first cyclisation of NAD$^+$ to cADPR and subsequent hydrolysis to ADPR$^{125-127}$, would result in slower kinetics compared hydrolysis alone, resulting in higher activity of cADPR. However, applying ADPR in the presence of equal concentrations of β-NAD, completely abolished calcium-signaling response, suggesting that β-NAD might act as an inhibitor of P2Y receptors. This was further supported in experiments using CD38 knock-out mice$^{95}$ provided by Frances Lund, where cADPR failed to cause a calcium transient, while the P2Y signaling through ADPR and other known P2Y agonist remained unaffected$^{94, 128}$. Furthermore, ADPR perfusion of TRPM2 knock-out beta cells had no effect on calcium release, further supporting the role of this channel as calcium release protein.

In summary, these studies establish that TRPM2 can function in the plasma membrane as a Ca$^{2+}$ influx channel and in intracellular Ca$^{2+}$ stores as a Ca$^{2+}$ release channel. It further adds another function of the primary TRPM2 agonist ADPR, which can not only serve as
a cytosolic second messenger directly, but additionally can act as an extracellular agonist for P2Y and adenosine receptors, which can signal through PLC and generate IP₃-mediated Ca²⁺ release. The extracellular function of ADPR links Ca²⁺ signaling to CD38 activity, since this ecto-enzyme is the primary source for ADPR from NAD⁺ and cADPR. Thus ADPR represents a multifunctional first and second messenger for Ca²⁺ signaling that by virtue of stimulating Ca²⁺ release and Ca²⁺ influx may have significant impact on Ca²⁺-dependent exocytosis. TRPM2 may directly contribute to Ca²⁺ influx and support concomitant depolarization of the plasma membrane, which would activate and/or sustain the activity of voltage-dependent Ca²⁺ channels. All three functions synergize to elevate [Ca²⁺]i and therefore can function to support insulin release from beta cells.

TRPM2’s function is limited to calcium release in dendritic cells
Dendritic cells (DC) play a key role in adaptive immunity. DC precursors travel from the bone marrow through the bloodstream, where they eventually migrate into any tissue to become resident immature dendritic cells. Immature DCs scan the surrounding environment for pathogens in order to phagocytose them and/or eventually capture and internalize antigens. Subsequent to internalization, cells migrate from peripheral tissue to regional lymph nodes where they meet with resting T-cells and present their antigen to naïve T-cells. During their migration, DCs undergo morphological and functional maturation. Furthermore, dendritic cells play a role in T-cell activation and regulation of B-cells and NK-cells (natural killer). In the event of antigen recognition and directed migration/localization, chemokine receptor signaling, followed by calcium signaling, plays a critical role in the recruitment of these highly controlled processes.

In this study it was demonstrated for the first time that the calcium-conducting channel TRPM2 is present in immature mouse dendritic cells. Surprisingly, however the function of TRPM2 in immature dendritic cells was clearly limited to calcium release. As a model to study dendritic cells, bone marrow-derived precursor cells were chosen and cultured with GM-CSF + IL-4 to force differentiation into immature dendritic cells. DC development is somewhat complex and precursors from both myeloid and lymphoid origin have been reported. Unlike hematopoietic lineages like T-cells, B-cells, neutrophils and monocytes, DC development derives from different sources and
comprises a large collection of subpopulations with different functions. DCs can also be differentiated out of monocytic cells, which interestingly express functional TRPM2 as a plasma membrane channel. Thus, within the progression of development, cells seem to lose plasma membrane-residing TRPM2, so that the channel function profile changes from calcium influx to calcium release. Furthermore, during this study it was observed, that throughout maturation, dendritic cells lose TRPM2 channel expression entirely, being absent in mature DCs (personal observation). Further studies should resolve this.

In order to investigate the physiological role of TRPM2, which possibly contributes to chemokine signal transduction in immature dendritic cells, studies were performed using different chemokines. LTB4, which is involved in T-cell activation and migration of DCs was clearly targeting the IP3 receptor only, as its effect were completely antagonized by heparin alone. Other mediators like ELC (Mip-3-alpha), which is involved in the mobilization of immature DCs to lymphoid organs, or SDF-1alpha, which targets the CXCR4 receptor and is also important for migration in response to chemokines, are currently under investigation.

Interestingly experiments using hydrogen peroxide, which targets multiple cellular processes in TRPM2 activation resulted in both calcium release and influx in DCs. Here oxidative stress-induced calcium influx due to hydrogen peroxide activated a channel different from TRPM2. A broad variety of channels conducting calcium like TRPC, TRPM7, TRPA1 and L-type calcium-channels have been reported to respond to reactive oxygen species (ROS). The current-voltage relationship of the H2O2-induced current was not linear and had similarities to TRPA1 or TRPC channels (data not shown). Both ROS-induced calcium release and ROS-mediated calcium influx are currently under investigation. To elucidate whether TRPM2 is involved in the transmission of signals caused by either reactive oxygen species or in the possible facilitation of maturation and chemo-tactic chemokine signaling, the TRPM2 knock-out mouse will be a valuable tool.
**TRPM2’s function is limited to a calcium influx in human neutrophils**

The study conducted in neutrophils demonstrates a comprehensive investigation of synergistic and antagonistic interactions of molecules known to influence TRPM2 activation in primary human neutrophils. Here it was shown that intracellular Ca\(^{2+}\) is required for ADPR-induced TRPM2 recruitment in primary human neutrophils with half-maximal potentiation observed at 300 nM [Ca\(^{2+}\)]. Furthermore, endogenous TRPM2 currents are very efficiently activated by ADPR in the low micromolar range in unbuffered Ca\(^{2+}\) conditions, representing a left-ward shift in sensitivity of about one order of magnitude compared to heterologously expressed channels. Similarly, TRPM2 can also be activated by cADPR and NAADP with 40-100-fold lower efficiency. In agreement with previous work 64, but in contrast to a study by Luckhoff and colleagues 86, intracellular cADPR, H\(_2\)O\(_2\) and NAADP synergize with subthreshold ADPR concentrations (100 nM) to trigger TRPM2 activation. Finally, it was establish that in human neutrophils, AMP and 8-Bromo-cADPR, suppress ADPR and cADPR-induced TRPM2 currents, respectively.

This study is the first detailed investigation of TRPM2 activity in response to ADPR and known co-activators of ADPR and inhibitors thereof in a primary cell system using K\(^+\)-based internal solutions. Ca\(^{2+}\) is well known to be an important co-activator of ADPR-induced TRPM2 currents 21, 86, 98-100. From the available data in overexpression systems, the EC\(_{50}\) values for [Ca\(^{2+}\)], range between 340 nM in Cs\(^+\)-based solutions 100 to about 40 nM in K\(^+\)-based solutions 98. A dose-response curve for intracellular Ca\(^{2+}\) has not been conducted in other cell lines or primary cell systems, although Heiner et al. 86 reported a two-point measurement of either low (10 mM EGTA) or high (1 μM) intracellular Ca\(^{2+}\) on ADPR-induced TRPM2 responses in human neutrophils using Cs\(^+\) as main intracellular ion. They showed that the EC\(_{50}\) for ADPR was about 1 μM in the presence of 1 μM [Ca\(^{2+}\)], which is similar to our data using unbuffered Ca\(^{2+}\) conditions and K\(^+\)-based internal solutions. Although it was surmised that [Ca\(^{2+}\)], under unbuffered conditions is likely to remain below 1 μM, it seemed that Cs\(^+\) does not significantly affect the ADPR-sensitivity of TRPM2 in neutrophils, unlike in HEK293 and Jurkat T cells cells 64. The data further show that internal Ca\(^{2+}\) co-activates TRPM2 currents with an EC\(_{50}\) of 300 nM at maximal ADPR concentrations. Therefore, the present Ca\(^{2+}\) dose-
response data, as well as data acquired in unbuffered conditions \(^9^8\) and the results reported by Heiner et al. \(^8^6\) together argue in favor of a picture where concentrations higher than 1 \(\mu M\) \(Ca^{2+}\) do not significantly potentiate ADPR effects. Thus, even high \([Ca^{2+}]_i\) by itself is not expected to activate TRPM2 currents at ADPR levels below 300 nM. Interestingly, although the overall ADPR concentrations in human neutrophils is around 5 \(\mu M\) \(^8^6\), the ADPR concentration in the vicinity of the channel seems to be below 300 nM, since raising intracellular calcium without disturbing intracellular basal ADPR levels does not cause TRPM2 activation.

Intracellular cADPR has been reported as a \(Ca^{2+}\)-release agent in various cell types \(^1^0^3\) and recent data suggest a role in \(Ca^{2+}\) influx \(^1^5^0\), which at least in part has been linked to TRPM2 activation \(^6^3, 6^4, 1^5^1\). In HEK293 cells overexpressing human TRPM2 channels, cADPR is quite inefficient in activating the channel with an \(EC_{50}\) of 700 \(\mu M\) in unbuffered \(Ca^{2+}\) and \(K^+\)-based conditions \(^6^4\). Jurkat T lymphocytes and human neutrophils, on the other hand, are at least 10-fold more sensitive to cADPR at otherwise identical experimental conditions \(^6^4\). Only when exposing HEK293 cells overexpressing rat TRPM2 channels to 40 °C does cADPR reach an \(EC_{50}\) value of around 60 \(\mu M\) \(^1^5^1\). Importantly though, the presence of both ADPR and cADPR increases the effectiveness of TRPM2 recruitment in both heterologous and endogenous expression systems. While this observation has not been quantified in great detail for Jurkat T cells, the synergistic effect of ADPR and cADPR cooperativity at subthreshold levels impresses with a 100-fold shift in \(EC_{50}\) for TRPM2 activation in HEK293 cells (from 12 \(\mu M\) to 90 nM for ADPR; \(^6^3\)) and a 15-fold shift in human neutrophils (from 44 \(\mu M\) to 3 \(\mu M\) for cADPR).

The ability of cADPR to activate TRPM2 currents may not be directly due to cADPR itself, but possibly to synergism with ADPR made available from other sources, such as contamination of the cADPR salt or ambient levels or metabolically produced ADPR from cADPR by cytosolic ADP-ribosyl cyclases \(^1^5^2\). Luckhoff and colleagues reported that in human neutrophils cADPR failed to activate TRPM2 currents even at 10 \(\mu M\) concentrations if the cADPR was pre-treated with pyrophosphatase to break down any ADPR contamination into ribose-5-phosphate and AMP \(^8^6\). ADPR contamination of the cADPR used in their study was estimated to be at 25%. The cADPR lot used in the present study had significantly lower contamination levels. HPLC data obtained from the
manufacturer (Sigma-Aldrich, USA) determined that the lot of cADPR had 94.5% purity, with 4.2% contamination of NAD$^+$ or nicotinamide and 1% contamination with other unidentified nucleotides, possibly including ADPR. This quantitative analysis is compatible with functional assays obtained. Assuming a 1% contamination of ADPR in the lot of cADPR that was used, a 100-fold concentration of cADPR should have similar efficacy of activating TRPM2 currents if this were solely due to ADPR contamination in the absence of any facilitatory action of cADPR. However, this is not the case, as the EC$_{50}$ for cADPR is around 40 $\mu$M rather than the expected 110 $\mu$M, indicating that cADPR synergizes with ADPR to enhance TRPM2 activity. This is further corroborated by adding subthreshold ADPR (100 nM) to increasing cADPR concentrations, which is sufficient to shift the EC$_{50}$ value about 15-fold from 44 $\mu$M to 3 $\mu$M, close to the apparent EC$_{50}$ for ADPR itself.

The relatively high contamination levels of cADPR with 25% ADPR may also account for a further discrepancy between this work $^{63,64}$ and previous results $^{86}$. The latter study did not observe any synergy of subthreshold ADPR (100 nM) and 10 $\mu$M cADPR, when cADPR was pre-treated with pyrophosphatases to remove the contaminating ADPR. This might be explained by the fact that such treatment will replace the contaminating ADPR with equal levels of its breakdown products. It was previously shown that, while ribose-5-phosphate does not interfere with TRPM2 activation by ADPR $^{63}$, AMP can inhibit ADPR-mediated activation of TRPM2 $^{63,64}$. Furthermore, the data presented here show that a 100-fold surplus of AMP is quite effective in suppressing TRPM2 currents in the presence of 1 $\mu$M ADPR. Thus, when degrading ADPR using the pyrophosphatase, AMP levels would increase in the cADPR sample to similar levels of the previous ADPR contamination. With an estimated contamination level of 25% by ADPR $^{86}$, cADPR very likely would have contamination levels of 25% AMP after pyrophosphatase treatment. Hence, using 10 $\mu$M of pre-treated cADPR would contain around 2.5 $\mu$M AMP, which might be sufficient to suppress any effect caused by the additionally added 100 nM ADPR. The presence of AMP in the pre-treated cADPR sample may also explain the absence of cADPR effects on TRPM2 observed previously in neutrophils $^{86}$, since the relatively high AMP contamination might prevent synergistic action with cellular ADPR that might have otherwise synergized with cADPR. As the cADPR antagonist 8-Bromo-
cADPR is also quite effective in suppressing TRPM2 activation by cADPR, one might infer that interference with either ADPR or cADPR prevents synergism of the two compounds and thus preventing any facilitatory effect on TRPM2 activation mediated through these molecules.

ADPR not only synergizes with cADPR to facilitate TRPM2 currents, but also with H_2O_2 and nicotinic acid adenine dinucleotide phosphate (NAADP), the latter being the most powerful Ca^{2+} release agent known to-date. H_2O_2 has widely been used as activator of TRPM2 for cells expressing this channel, although it should be kept in mind that H_2O_2 also gives rise to a membrane-delimited Ca^{2+}-permeable cation current that is not linked to TRPM2 but most likely due to lipid peroxidation. Nevertheless, while neither 100 µM H_2O_2 nor 100 nM ADPR alone are sufficient to cause TRPM2 activation in human neutrophils, the combination of the two causes TRPM2 currents that are comparable to perfusion with 1 µM ADPR alone, both in terms of amplitude and kinetics of activation. NAADP is known to be a potent Ca^{2+}-release agent in sea urchin eggs, acting in the low nanomolar range. NAADP does not involve inositol 1,4,5 trisphosphate (IP_3) receptors, but is thought to be due to a novel NAADP receptor in sea urchins. In some eukaryotic cell types, NAADP seems to act on ryanodine-receptor sensitive stores. Furthermore, data from this laboratory implicate TRPM2 as a novel target for NAADP in HEK293 cells overexpressing TRPM2 and native TRPM2 channels in Jurkat T cells, albeit at 70-fold lower potency than ADPR. This is also observed in human neutrophils, where TRPM2 currents are activated by NAADP with an EC_{50} of 95 µM. In addition, NAADP seems to facilitate TRPM2 currents when combined with subthreshold ADPR (100 nM), but only within the narrow concentration range of 0.3 µM to 30 µM NAADP. ADPR does not seem to shift the dose-response behavior of NAADP at higher concentrations of NAADP. This behavior may be due to the bell-shaped dose-response curve for NAADP-induced Ca^{2+}-release observed in other cell systems, where optimal Ca^{2+}-release is elicited by NAADP concentrations between 10 nM and 1 µM, but concentrations above 100 µM NAADP abolish Ca^{2+} release. Thus, lower NAADP concentrations may cause additional Ca^{2+}-release, which in conjunction with subthreshold ADPR facilitates TRPM2 currents in a narrow concentration window. This
facilitatory action mediated by Ca$^{2+}$ would then be lost at higher NAADP concentrations even in the presence of subthreshold ADPR$^{155-157}$.

In conclusion, TRPM2 currents measured in primary human neutrophils are sensitive to internal ADPR levels at physiologically relevant concentrations$^{86,117}$, with intracellular Ca$^{2+}$ acting as a mandatory and dose-dependent co-activator of the channel. While cADPR and NAADP recruit TRPM2 in neutrophils in the low micromolar range, these agonists may not represent primary or singular activators of TRPM2, but rather work in synergy with ADPR, thus regulating the efficacy and the sensitivity of TRPM2 channels in conjunction with internal Ca$^{2+}$.

**Influence of CD38 in the regulation of TRPM2 in mouse neutrophils**

Taking advantage of the availability of different knock-out mice, experiments similar to those made with human neutrophils were conducted in mouse neutrophils to assess the biophysical properties of TRPM2 currents induced by ADPR. Mouse neutrophils, which were similar in morphology to human neutrophils, though slightly smaller, displayed almost identical TRPM2-channel properties as those in human neutrophils, exhibiting half-maximal activation in the mid-nano molar range (EC$_{50}$ mouse 500 nM; EC$_{50}$ human 300 nM). Perfusion of neutrophils isolated from TRPM2 knock-out mice with ADPR did not evoke any currents, which demonstrated that the relatively large currents induced by ADPR are entirely mediated through TRPM2 and not by any unidentified channel type. Furthermore, neutrophils isolated from mice deficient in the ectoenzyme CD38 (described above) were investigated. CD38 represents a key factor regulating calcium release and influx required for neutrophil chemotaxis$^{69}$. In addition, all three products (ADPR, cADPR and NAADP) of CD38 enzyme activity are potential activators of TRPM2$^{85}$. Whether these nucleotides traverse the membrane passively or are actively transported still remains to be elucidated. Interestingly, TRPM2 channels were functional in neutrophils isolated from CD38-deficient mice, however investigating TRPM2 in cd38$^{-/-}$ mouse pancreatic beta cells showed absence of ADPR-induced currents (data not shown). Further studies are needed in order to understand the physiological mechanisms of this complex circuit of nucleotide signaling.
**TRPM2-mediated calcium influx in mouse monocytes**

Monocytes, which represent about 5-10% of peripheral blood leukocytes\(^{158}\), derive from myeloid precursors. They travel through the bloodstream and migrate into tissue. With a relatively short lifespan of 1-3 days, monocytes potentially differentiate into tissue specific macrophages or dendritic cell subsets\(^{159}\). Monocytes, like macrophages, neutrophils and eosinophils, play a role in innate immunity and respond to inflammation with a so-called respiratory burst due to their NADPH oxidase activity\(^{160}\) producing reactive oxygen species (ROS). TRPM2 is known to be activated by ROS through activation of the PARP/PARG pathway and possibly ADPR release from mitochondria\(^{67}\). It is believed that ROS primarily facilitate pathogen killing upon phagocytosis\(^{161}\). Recently, ROS emerged as signaling molecules in the recruitment of inflammatory and immune responses. In order to evaluate the role TRPM2 in signaling processes of inflammation, the group of Yasuo Mori created a TRPM2 knock-out mouse. In their study using the human monocytic cell line U937 they demonstrate, that calcium influx through TRPM2 is necessary to trigger calcium-dependent tyrosine kinase Pyk2. This leads to activation of Erk through Ras and eventually elicits nuclear translocation of NF-kB\(^{162}\) which is essential for the production of interleukin-8 (CXCL8). In addition they demonstrate that the production of CXCL2, a mouse CXCL8 homologue, is impaired upon hydrogen peroxide stimulation in mice deficient of TRPM2. Furthermore, inducing colitis through dextran sulfate sodium (DSS) as a model of inflammation\(^{163}\) that involves ROS\(^{164}, \ 165\), they demonstrate that production of CXCL2, neutrophil infiltration and ulceration was significantly attenuated in TRPM2\(^{-/-}\) mice.

This is the first demonstration of the involvement of TRPM2 in an inflammatory process, promoting the channel as a potential target for treatment of inflammatory diseases. For this study, the biophysical characterization of primary mouse monocytes from blood in wild-type and TRPM2-deficient mice was conducted. From a biophysical point of view, the activation of TRPM2 though ADPR in monocytes reveals similar properties as those described for neutrophils. The currents were linear and developed rapidly and were proportional to cell size. It was clearly demonstrated that TRPM2-deficient mice lack distinct ADPR-induced current development. Unlike in human neutrophils, internal perfusion of hydrogen peroxide was sufficient to trigger the characteristic current in
neutrophils from wild-type mice and absent in neutrophils from knock-out mice. Whether this might be due to different oxidative-burst or ROS-release activities in neutrophils or displaying a higher threshold to ROS than monocytes, which would serve as a protection mechanism preventing intrinsic TRPM2 activation, is still controversial. Other factors contributing to the differences include variations in species, nucleotide metabolism or TRPM2 location\textsuperscript{160,166}. The question arose whether other calcium-influx pathways would be affected in TRPM2 KO mice to compensate for the loss of TRPM2. Therefore, the store-operated and highly selective calcium channel CRAC\textsuperscript{19,29} and the nucleotide inhibited cation channel TRPM7\textsuperscript{51} were both measured in wild-type and TRPM2\textsuperscript{-/-} monocytes. No significant difference could be observed. Therefore Yamamoto et al. demonstrate for the first time since the identification of TRPM2 its pathophysiological relevance in a model of inflammatory disease. Furthermore it brings the known pathway of nuclear factor B recruitment into the context of TRPM2 activation\textsuperscript{167}.

**AMP inhibits receptor-mediated calcium release through unknown mechanism**

During the work on TRPM2 acting as a release channel the possibility was considered that production of intracellular ADPR might occur via stimulation of classical receptor pathways\textsuperscript{168-171}. From the biophysical experiments it was known that AMP was able to inhibit the effect of ADPR by possibly antagonizing the reaction at the NUDT-9h domain\textsuperscript{21}. It was presumed that high concentrations (up to 1 mM) of AMP would be sufficient to inhibit calcium release caused by any metabolized or intrinsically released ADPR. Experiments, where diverse receptors were activated and different checkpoints of G-protein-signaling cascade were inhibited, showed that most of theses pathways eventually lead to the production of IP\textsubscript{3}. Interestingly, external application of ADPR on INS-1 cells (rat insulinoma monoclonal) triggered a calcium release that was neither antagonized by inhibiting the G-protein nor the IP\textsubscript{3} receptor itself. The fact that only co-perfusion with heparin and AMP was able to suppress this release signal lead to the conclusion that ADPR would somehow be metabolized and subsequently act on TRPM2 located in the endoplasmic reticulum. Interestingly, the PLC inhibitor U73122 was able to abolish the ADPR-induced signal. This was surprising, as inhibition of G-proteins did not inhibit the signal, which is upstream of PLC. One explanation would be that the inhibitor U73122 disrupts the membrane integrity of the cell such that unknown factors
of this novel mechanism are impaired as well. In fact experiments were hard to perform in the presence of U73122, as the membrane integrity indeed was often not suitable for electrophysiological recordings.

In INS-1 cells the ADPR-induced signal activates two classes of nucleotide receptors, namely P2Y- and Adenosine Receptors. Here it was found that the AMP-sensitive calcium release signal was produced through P2Y receptor, whereas adenosine receptors clearly only lead to the production of IP$_3$. This novel P2Y-related pathway was only seen in INS-1. Primary cells did not express Adenosine receptor and ADPR-induced signals were entirely mediated by IP$_3$. When taking a closer look at the effects of AMP, it was found, that AMP alone was capable of antagonizing calcium release induced by multiple cell surface receptor agonists. It seemed to affect a ubiquitous mechanism independent of the cell system investigated, as it was observed in cell lines and primary cells, in mouse rat and human, as well as with different classes of agonist. This mechanism shared the production of IP$_3$, causing subsequent calcium release. Interestingly, the inhibitory effect was effective at low µM concentrations (1-10 µM), which seems to be at a physiological range, as AMP is a breakdown product of ADPR, the latter being at levels in the range of 5-70 µM$^{117}$. Furthermore there is the mitochondrial pyrophosphatase activity of NUDT9$^{172}$ and breakdown products of cADPR$^{86}$ all produce AMP, thus strengthening the physiological relevance of this finding. The data obtained in the INS-1 cells indicated that the action of AMP was distinctly different from that of a simple inhibition of IP$_3$ receptors, as both heparin and AMP were required for signal silencing. Another preliminary result was that the effect of AMP could be entirely substituted through capsazipine, which is an antagonist to TRPV1. Transient receptor potential valinoid 1 is a nonselective cation channel conducting calcium, which has been stated as an important factor in peripheral nociception$^{173}$ as well as functionally acting as a calcium release channel$^{78}$. Both mechanisms of AMP and capsazipine are not yet clearly understood. To gain more insight into this finding, future work will need to be carried out in primary cells, where the role of TRPV1 could possibly implicate it as a target of G-protein-coupled receptor pathways in the transduction of pain sensation.
SUMMARY

TRPM2, a member of the transient receptor potential family, is a widely expressed Ca\textsuperscript{2+}\,-permeable, non-selective cation channel that is specifically activated by ADP-ribose (ADPR) at its unique enzymatic pyrophosphatase domain. The physiological role of TRPM2, in the context of calcium signaling, is still uncertain but has been linked to apoptosis. In this study, a novel function has been resolved extending TRPM2’s role beyond being a plasma membrane channel. In pancreatic beta cells, TRPM2 not only performs as a calcium conducting plasma membrane channel, but also releases calcium from intracellular calcium stores upon stimulation with intracellular ADPR. Furthermore, ADPR, as well as cADPR, can act as an extracellular receptor agonist activating P2Y and adenosine receptors, thus evoking further Ca\textsuperscript{2+} release activity through IP\textsubscript{3} production. Thus, ADPR and TRPM2 represent multimodal signaling elements that regulate Ca\textsuperscript{2+} mobilization in beta cells where calcium regulation plays a crucial part in the mechanism of insulin release. Interestingly, in bone marrow-derived dendritic cells, where TRPM2 is highly expressed, its function is entirely limited to being a calcium release channel. In contrast, in human neutrophils TRPM2’s function is limited to its role as a plasma membrane ion channel. Conceivably, the differential and cell-type specific localization of TRPM2 might generate new insights into its physiological function. A detailed study on channel regulation was conducted in primary human neutrophils. It was demonstrated that the channel is synergistically regulated by cADPR, NAADP and hydrogen peroxide. The biophysical properties of agonists and antagonists of TRPM2 as seen in overexpression systems were demonstrated to be largely compatible with the biophysical properties of TRPM2 currents measured in human neutrophils, although agonists generally exhibited higher potencies of TRPM2 in primary cells. Similar efficacies of ADPR were resolved in mouse neutrophils, where cells isolated from cells deficient of the ADPR-producing ecto-enzyme CD38 did not show any difference in current activation compared to wild-type. In collaboration with the laboratory of Yasuo Mori, Kyoto, Japan, a biophysical characterization of TRPM2 was conducted using blood-derived monocytes isolated from wild-type and TRPM2 knock-out mice. Here, the functional ablation of TRPM2 currents was demonstrated utilizing the knock-out mouse, further revealing insights into the patho-physiological role of TRPM2-deficiency in
immune function. Ongoing and future work will further unravel TRPM2’s place in the complex network of calcium-signaling transduction mechanisms and its potential underlying malfunctions in inflammatory diseases. Finally, it was demonstrated that the TRPM2-antagonist adenosine monophosphate also acts as a potent inhibitor of G-protein-coupled receptor-stimulated calcium release. The action of AMP in inhibiting the IP$_3$-mediated release may represent a ubiquitous mechanism, though the details underlying this phenomenon still require further elucidation.

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APPENDIX

Abbreviations

ADP  adenosine diphosphate
ADPR  adenosine 5’-diphosphoribose
AMP  adenosine mono phosphate
ATP  adenosine triphosphate
A1/2/3  adenosine receptor
Ca^{2+}  calcium
cADPR  cyclic adenosine diphosphate ribose
CAM  calmodulin
Cch  charbachol
cd38  cluster of differentiation 38
CRAC  calcium release activated current
CRACM  calcium release activated current modulator
CRI-G1  rat pancreas islet tumor cell line
CXCR  CXC chemokine receptor
DAG  diacylglycerol
DC  dendritic cell
DMEM  Dulbecco’s modified Eagle’s medium
DMSO  Dimethylsulfoxide
EC_{50}  effective concentration 50%
EDTA  Ethylenediaminetetraacetic acid dipotassium salt dihydrate
EGTA  ethylene glycol tetraacetic acid
ERK  extracellular signal regulated kinase
GDP-β-S  Guanosine 5’-β-[thio]diphosphate trilithium salt
HBSS  Hank’s balanced salt solution
HEK  human embryonic kidney
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid
HPLC  high performance liquid chromatography
IC_{50}  inhibitory concentration 50%
IL-4  interleukine 4
INS  insulinoma cell line
IP_{3}  inositol tris phosphate
IS  immunological synapse
I_{CRAC}  actual calcium release activated current
KO  knock-out
K_{d}  dissociation constant
LTB4  Leukotrien B4
M  Mol/Liter
MagNuM  Magnesium nucleotide regulated metal ion
mg  Milligram
mm  Millimeter
mM  Millimol/Liter
ml  Milliliter
NAADP  Nicotinic acid adenine dinucleotide phosphate sodium salt
NAD  β-nicotineamide dinucleotide
NADPH  Nicotinamide adenine dinucleotide phosphate
NF-κB  nuclear factor-kappa B
ng  nanogram
nm  nanometer
nM  nanomol/Liter
NUDT9  Nudix (nucleoside diphosphate linked moiety X)-type motif 9
NUDT9-h  Nudix (nucleoside diphosphate linked moiety X)-type motif 9 homology
pA  pico Ampere
PARG  poly ADP-Ribose glycohydrolase
pA/pF  currents in Ampere normalized on cell size in Farad (capacitance)
PARP  poly ADP-Ribose polymerase
PBS  phosphate buffer saline
pF  pico Farad
PLC  phospholipase C
PMCA  plasma membrane calcium ATPase
pS  pico Siemens
PTPL1  protein tyrosine phosphatase
Pyk2  calcium dependent tyrosine kinase
P2Y  purinergic receptor
RAS  small GTPases
RINm5F  rat insulinoma cells
RNA  Ribonucleic acid
ROS  reactive oxygen species
RPMI  named after Roswell Park Memorial Institute
scrambled  nonmatching sequence
SCID  severe combined immune deficiency
SERCA  sarco/endoplasmic- reticulum calcium ATPase
siRNA  small interfering RNA
STIM  stromal interaction molecule
TCR  t-cell receptor
TRPA  transient receptor potential ankyrin
TRPC  transient receptor potential canonical
TRPM  transient receptor potential melastatin
TRPN  transient receptor potential no mechanoreceptor potential C
TRPV  transient receptor potential vanilloid
WT  wildtype
8-Br-ADPR  8-Bromo- adenosine 5’-diphosphoribose
8-Br-cADPR  8-Bromo cyclic adenosine diphosphate ribose
µg  Microgram
µl  Microliter
µM  Micromol/Liter
µm  Micrometer
[Ca^{2+}]_I  cytosolic free calcium concentration
Publications

TRPM2-mediated \( \text{Ca}^{2+} \) influx induces chemokine production in monocytes that aggravates inflammatory neutrophil infiltration

Synergistic Regulation Of Endogenous TRPM2 Channels By Adenine Dinucleotides In Primary Human Neutrophils
Ingo Lange, Reinhold Penner, Andrea Fleig & Andreas Beck, accepted, Cell Calcium

ADP-Ribose is a Multimodal Agonist for Purinergic Receptors and TRPM2 Channels in the Plasma Membrane and Intracellular Stores of Beta Cells
Ingo Lange, Santiago Partida-Sanchez, Shinichiro Yamamoto, Yasuo Mori, Andrea Fleig & Reinhold Penner, submitted

Activation of the \( \text{Ca}^+ \)-permeable non-selective cation channel TRPC6 but not TRPM2 channels in murine dendritic cells during oxidative stress
Adriana Sumoza-Toledo, Ingo Lange, Harivadan Bhagad, Frances Lund, Andrea Fleig, Reinhold Penner, Santiago Partida-Sanchez, in preparation

TRPM2 activation is required for migration of dendritic cells in response to CXCR4 and CCR7 chemokine receptors
Adriana Sumoza-Toledo, Ingo Lange, Harivadan Bhagad, Andrea Fleig, Reinhold Penner, Yasuo Mori, Santiago Partida-Sanchez, in preparation
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