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Conserved cysteine residues in the pore region are obligatory for human TRPM2 channel function

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Abstract

TRPM2 proteins belong to the melastatin-related transient receptor potential or TRPM subfamily and form Ca²⁺-permeable cationic channels activated by intracellular adenosine diphosphoribose (ADPR). The TRPM2 channel subunit, like all its close relatives, is structurally homologous to the well-characterized voltage-gated potassium channel subunits, each containing six transmembrane segments and a putative pore loop between the fifth and sixth segments. Nevertheless, the structural elements determining the TRPM2 channel functions are still not well understood. In this study, we investigated the functional role of two conserved cysteine residues (at positions 996 and 1008) in the putative pore region of the human TRPM2 by site-directed mutagenesis combined with electrophysiological and biochemical approaches. Expression of wild type hTRPM2 channels in HEK293 cells resulted in robust ADPR-evoked currents. Substitution of cysteine with alanine or serine generated mutant channels that failed to be activated by ADPR. Furthermore, experiments by Western blotting, immunocytochemistry, biotin labelling, and co-immunoprecipitation techniques showed no obvious changes in protein expression, trafficking or membrane localisation, and the ability of interacting with neighbouring subunits that is required for channel assembly. Co-expression of wild type and mutant subunits significantly reduced the ADPR-evoked currents; for combination of wild type and C996S mutant subunits, the reduction was approximately 95%, indicating that incorporation of one or more non-functional C996S subunits leads to the loss of channel function. These results taken together suggest that the cysteine residues in the pore region are obligatory for TRPM2 channel function.

Keywords

TRPM2; ADP-ribose; cysteine residue; site-directed mutagenesis; Western blot; patch-clamp recording

Introduction

Transient receptor potential (TRP) channels are a very large family of integral membrane proteins, which form cationic channels with diverse profiles with regard to their mechanisms of activation, electrophysiological and biophysical properties, and physiological functions (3, 4, 22, 28). Based on structural relatedness, mammalian TRP proteins have been categorised into six subfamilies: TRPC (canonical), TRPV (vanilloid receptor-related), TRPM (melastatin-related), TRPP (polycystin-related), TRPML (mucolipin-like), and TRPA (ankyrin) (3, 4, 9, 21, 22). TRPM2, formerly known as TRPC7 (24) or LTRPC2 (27), forms Ca²⁺-permeable cationic channels that are specifically activated by intracellular

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adenosine diphosphoribose (ADPR) (27, 32). A number of factors including nicotinamide adenine dinucleotide, H_2O_2 , cyclic ADPR and intracellular Ca^{2+} have been reported to activate TRPM2 channels independently of or synergistically with ADPR (8, 10, 17, 20, 32, 34). TRPM2 mRNA is highly expressed in the brain and also detected in many other tissues including the heart, placenta, lung, kidney, spleen and ovary (27, 33). TRPM2 or TRPM2-like functional responses have been documented in striatal neurons (12), U937 monocyte and Jurkat cells (27, 32), neutrophil (10), insulinoma cells (8, 13) and microglia (18). ADPR and other endogenous activators or synergising ligands and TRPM2 channels are thought to represent an intrinsic mechanism of providing Ca^{2+} entry into cells and regulating cellular functions (32) or causing cell death by disrupting cellular homeostasis in response to changes in cellular redox states (8).

TRPM2 proteins comprise an ion channel core domain and cytoplasmic N- and C- termini (Fig. 1). While no specific functional roles have been designated for the N-terminus (residues 1–761 in hTRPM2), a major distal part (residues 1236–1503; termed NUTD9-H domain) of the C-terminus which encompasses residues 1049–1503, is homologous to the NUTD9 proteins, ADPR pyrophosphatases belonging to the nudix hydrolase family, and contains the binding site for ADPR (19, 27). The ion channel domain (residues 762–1048) is composed of six transmembrane (6TM) segments (S1–S6) and a pore loop between the S5 and S6, similar to the voltage-gated potassium channels and other six 6TM channels including cyclic nucleotide gated channels. Nevertheless, sequence analysis of these closely related channels or even for the TRP channel family members has led to the identification of sequence differences as well as sequence homology. The functional implications remain yet to be established. Sequence alignment of the pore regions of the TRP superfamily indicates that, unlike their close relatives of the TRPC and TRPV subfamilies, all the members of the TRPM subfamily contain a pair of cysteine residues (positions 996 and 1008 in the human TRPM2 subunit) of which the first is completely conserved within the TRPM subfamily and the second is present at slightly different positions (Fig. 1). In this study, we combined site-directed mutagenesis with biochemical and electrophysiological approaches to investigate the potential role of these conserved cysteine residues in the TRPM2 channel function. Substitution of cysteine with either alanine or serine generated mutant channels which were functionally unresponsive to ADPR. Furthermore, biochemical experiments showed that loss of the channel function was not due to effects on protein expression, membrane trafficking or localisation. The mutant subunits also showed normal interaction with neighbouring subunits. However, co-expression of wild type with mutant subunits markedly reduced the ADPR-evoked currents. These results taken together suggest an obligatory role in channel function of cysteine residues in the pore region of TRPM2.

Materials and Methods

Chemicals, constructs, cell culture and transfection

All the chemicals and reagents were purchased from Sigma unless otherwise specified. The cDNA encoding the human TRPM2 protein with an N-terminal FLAG tag was a kind gift from AM Scharenberg (Washington University, Seattle, USA). The plasmid encoding the hTRPM2 subunit with a C-terminal EE tag was constructed as follows. The hTRPM2 and tag sequences were amplified by PCR using the following pair of primers: 5'-ATG GAG CCC TCA GCC CTG AGG AAA GC-3' (forward primer) and 5'-CTA TTC CAT TGG CAT GTA TTC GTA GTG AGC CCC GAA CTC AG-3' (reverse primer). The PCR amplicon was inserted into the vector pCR2.1 (Invitrogen), and the fragment between *HindIII* and *EcoRV* was excised and subcloned into pcDNA 3.1/*myc*-His vector (Invitrogen) between *HindIII* and *PmeI* sites. The coding sequences were verified by sequencing. Both FLAG-tagged and EE-tagged constructs were used in this study, and no noticeable differences were seen in their functional properties and therefore data where appropriate

were pooled. Point mutations were introduced in the EE-tagged hTRPM2 construct using the QuikChange site-directed mutagenesis protocol (Stratagene) and verified by sequencing.

Human embryonic kidney cells (HEK293) were used in this study to express wild type and mutant TRPM2 proteins. Cells were maintained in Dulbecco's modified Eagle medium supplemented with 2 mM glutamine and 10% heat-inactivated foetal bovine serum (all from Invitrogen) at 37°C under 5% CO₂ humidified conditions. Transient transfection of HEK293 cells with plasmids was carried out using lipofectamin2000 (Invitrogen) according to the manufacturer's instructions.

Electrophysiological recordings

Cells were grown in 35 mm petri-dishes and transfected with 1 µg hTRPM2 plasmid (and 0.1 µg green fluorescent protein (GFP) plasmid). Transfected cells were seeded on glass cover-slips 12–24 hr after transfection and used in the following 24–72 hr. Whole cell recordings were made at room temperature using an Axopatch 200B amplifier and analysed using pCLAMP9 software (Axon Instruments). Patch electrodes with a resistance of 4–6 MΩ were fabricated from borosilicate glass capillaries (World Precision Instruments). Cells were held at –40 mV and voltage ramps from –120 mV to 80 mV with 1 s duration were applied every 5 or 10 s. Extracellular solutions contained: 147 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES and 13 mM glucose (Na⁺ solution), or 149 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES and 13 mM glucose (K⁺ solution), or 110 mM CaCl₂, 10 mM HEPES and 13 mM glucose (K⁺ solution). Intracellular solution contained 147 mM NaCl, 50 µM EDTA, 1 mM MgCl₂, 10 mM HEPES, and 1 mM Na₂ATP. All the solutions were maintained pH7.3 with NaOH and 300–315 mosm/l. ADPR stock solution (100 mM) was made in intracellular solution, kept at –20°C in aliquots, and diluted to the indicated concentrations in intracellular solution prior to use. Flufenamic acid (FFA) was freshly made as a 1 M stock solution in dimethyl sulfoxide (DMSO) and diluted in extracellular solution to a final concentration of 0.5 mM prior to application; there was no detectable effect of DMSO as previously reported (11). FFA was applied for 0.5–1 min via a rapid solution changer RSC-160 (Biologic Science Instruments, France). All the data were presented as mean ± SEM, and statistical analysis was carried out using Student's *t* test where appropriately.

Immunocytochemistry

Cells were prepared as described above for electrophysiological recordings without addition of GFP plasmid in the transfection. Immunocytochemistry was performed using protocols previously detailed (30). Briefly, we used mouse monoclonal anti-EE primary antibodies (1:1000 dilution; Babco, Richard, USA), and a FITC-conjugated anti-mouse IgG secondary antibody (1:200 dilution, Sigma).

Co-immunoprecipitation, biotinylation and Western blotting

Cells were prepared in T25 flasks (~3 × 10⁶ cells) and transfected with 3 µg plasmid as described above. For each flask, cells were collected into 1 ml chilled phosphate buffered saline (PBS) 24 hr after transfection. For co-immunoprecipitation experiments, cells were pelleted at 4°C by centrifugation and lysed at 4°C for 30 min in 200 µl lysis buffer containing 50 mM Tris-HCl pH8.0, 150 mM NaCl, 2 mM EGTA, 1% Triton X-100, 5% glycerol supplemented with a cocktail of protease inhibitors (Roche). After clearing by centrifugation at 4°C for 10 min, the supernatant was mixed with 20 µl pre-equilibrated Ezview red anti-FLAG M2 affinity gel beads and agitated at 4°C for 2 hr. The beads were collected by centrifugation at 8200×*g* for 30 s, kept on ice after removing the supernatant, and washed three times, each time by suspending in 1 ml lysis buffer and centrifuging at 8200×*g* for 30 s. After the final wash, the immunoprecipitated samples (beads) were re-

suspended in 40 μ l electrophoresis sample buffer containing 50 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% glycerol, 100 mM DTT, 0.05% bromophenol blue, boiled for 5 min and centrifuged at 8200 $\times g$ for 30 s.

For biotin labelling experiments, HEK293 cells were washed 3 times with PBS 24 hr after transfection and incubated in 1 mg/ml sulfo-NHS-biotin (Pierce) for 30 min at 4°C. The cells were then washed three times in PBS containing 50 mM glycine, and lysed with lysis buffer (see above) for 30 min at 4°C. Biotin-conjugated cell surface proteins were purified with streptavidin-agarose. Affinity-purified protein complexes were denatured as described above for co-immunoprecipitation protein samples.

Western blotting was performed at room temperature. Protein samples were resolved on 8% SDS-PAGE gels and transferred to nitro-cellular membranes under semi-dry transfer conditions (Biorad) for 1 hr (constant current, 1 mA/cm² membrane). After blocking for 1 hr with 5% non-fat milk made in TBST solution (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20), the membranes were incubated for 1 hr with primary rabbit anti-EE antibody (1:2000 dilution; Bethyl, Texas, USA), and washed three times in TBST solution for 5 min. The membranes were then incubated for 50 min with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2000 dilution; Santa Cruz), and washed three times in TBST solution as before. Proteins were visualized using supersignal west pico chemiluminescent substrates according to the manufacturer's protocol (Pierce) and captured on X-ray films.

Results

Functional expression and properties of wild type hTRPM2 channel

Whole cell current recordings were carried out, following transient transfection of HEK293 cells with TRPM2 cDNA, to confirm functional channel expression. Our preliminary experiments showed that the time course and the amplitude of the current responses were ADPR concentration-dependent which is consistent with previous reports (17, 27). Thus, a supermaximal concentration of ADPR (1 mM) was used throughout this study in order to obtain maximal functional responses. Fig. 2A shows the current responses of a cell expressing wild type TRPM2 channels to voltage ramps from -120 mV to 80 mV (left panel), and plots the current amplitudes at membrane potentials of -80 mV and +80 mV (right panel). Upon breaking into whole-cell configuration, large currents appeared which reached a maximal level within 10 s. Currents exhibited typical linear current-voltage (I/V) relationships (Fig. 2B) with a reversible potential close to zero (-1.3 ± 0.2 mV, $n = 5$), indicating that they were elicited by non-selective cationic channels. The currents varied from approximately 0.5 nA to more than 10 nA with a mean peak current amplitude of 5.0 ± 0.6 nA ($n = 18$) at a membrane potential of -80 mV. Such robust currents were never detected either in the TRPM2 channel expressing cells when recording using intracellular solution without ADPR (left panel in Fig. 2C) or in the non-transfected cells using intracellular solution containing ADPR (Fig. 3B). To further confirm that the ADPR-evoked currents were mediated by the TRPM2 channels, we tested flufenamic acid, a TRPM2 channel antagonist (11), and also the sensitivity to calcium (20). As illustrated in Fig. 2B, flufenamic acid (0.5 mM) rapidly and almost completely inhibited the ADPR-evoked currents (middle panel in Fig. 2C). The inhibition was independent of the membrane potential and irreversible during washing for 5 min (Fig. 2B). Upon changing extracellular solution containing no calcium (replaced with an equal molar concentration of barium) to extracellular solution containing 2 mM calcium, the ADPR-evoked currents were remarkably increased (right panel in Fig. 2C). These results clearly indicate that the ADPR-evoked currents are the result of activation of functionally expressed TRPM2 channels and

confirm that the electrophysiological and pharmacological properties observed here are similar to those reported by other groups (11, 17, 20, 27).

Effects of mutating C996 and C1008 on current responses to ADPR

To explore the potential functional role of cysteine residues at positions 996 and 1008 in the pore region, we firstly replaced these amino acid residues individually with alanine. In contrast to the robust ADPR-evoked currents observed following expression of the wild type channels, cells expressing either C996A or C1008A mutant channels failed to respond to ADPR (Fig. 3B). We then introduced serine substitutions, because we reasoned that this manipulation would produce the smallest structural alteration, in that it changed an SH- for an OH- group. Surprisingly, ADPR also either failed to elicit currents or only evoked very small currents in cells expressing these mutant channels (Fig. 3A and Fig.3B).

Whole-cell currents were also recorded from cells expressing wild type, C996S or C1008S mutant channels in extracellular solutions containing K^+ or Ca^{2+} as the major cations. In extracellular K^+ and Ca^{2+} solutions, ADPR elicited substantial currents in cells expressing wild type channels, but no current or very small currents in cells expressing C996S or C1008S mutant channels (Fig.3C). To rule out the possibility that loss of channel function was due to a non-specific mutational effect, we substituted alanine into a non-conserved glutamic acid residue (E994), one residue away from C996 (Fig.1). Expression of the E994A mutant channels, like the wild type channels, gave rise to robust ADPR-evoked currents (-6.1 ± 1.3 nA, $n = 5$), which were blocked by FFA ($98.0 \pm 1.3\%$, $n = 5$).

Effects on protein expression and membrane localisation

The loss of channel function as a result of mutating these cysteine residues could be attributed to mutational defects in either protein expression or protein trafficking or both. To investigate these possibilities, we performed Western blotting, immunostaining and biotin labelling experiments. Fig.4 shows the representative results. Clearly, there was no obvious difference in the expression levels of wild type or mutant subunit proteins (C996A, C996S, C1008A and C1008S) (Fig.4A). Furthermore, immunoreactivity for wild type, C996S and C1008S mutant subunits was similar, showing dominant clustering around the plasma membrane (Fig.4B). In addition, the biotin labelling experiments indicate that the levels of biotinylated TRPM2 protein at the plasma membrane were not greatly changed for wild type and mutant subunits (Fig. 4C). Taken together, these results suggest that the mutation of C996 or C1008 did not significantly alter the protein expression, the trafficking of the protein, or the membrane localisation and therefore does not account for the loss of channel activity.

Co-expression of mutant subunits with wild type subunit

TRPM2 channels are thought to form as tetramers, in analogy to voltage-gated potassium and other 6TM channels. The introduction of mutations in individual subunits could impair the subunit interactions which are required for the formation of functional channel complexes. To address this possibility, we co-expressed EE-tagged wild type, C996S or C1008S subunits with FLAG-tagged wild type subunits, and performed co-immunoprecipitation. As shown in Fig.5A, co-expression did not change the expression levels of either the EE-tagged wild type, C996S or C1008S mutant subunit proteins (top panel) or the FLAG-tagged wild type subunit proteins (middle panel). The amount of EE-tagged wild type, C996S and C1008S mutant subunit proteins, pulled down with the co-expressed FLAG-tagged wild type subunit proteins using an anti-FLAG antibody, was similar (bottom panel). Similar results were also seen in reciprocal co-immunoprecipitation experiments using an anti-EE antibody (data not shown). Taken together, these results

indicate that C996S and C1008S mutant subunits interact with neighbouring wild type subunits to the same extent as wild type subunits interact with each other.

We next examined the functional effects of C996S and C1008S mutant subunits by recording the ADPR-evoked currents from cells expressing wild type subunit alone, wild type subunit co-expressed with C996S subunit, or wild type subunit co-expressed with C1008S subunit. Fig.5 shows examples current recordings. A significant reduction in ADPR-evoked current amplitude was observed when wild type subunit was co-expressed with either C996S or C1008S mutant subunit. The mean amplitude of ADPR-evoked peak currents was 0.3 ± 0.06 nA ($n = 8$) from cells co-expressing wild type and C996S subunits, 1.2 ± 0.2 nA ($n = 11$) from cells co-expressing wild type and C1008S subunits and 4.7 ± 0.5 nA ($n = 13$) from cells expressing wild type subunit alone (Fig.5). The average ADPR-evoked currents were reduced by 94% and 75% as a result of co-expression of wild type subunit with C996S mutant subunit, and co-expression of wild type subunit with C1008S mutant subunit, respectively.

Discussion

The main finding from this study, which combines site-directed mutagenesis with biochemical and electrophysiological approaches, is that the conserved cysteine residues in the pore region are obligatory for normal TRPM2 channel function.

Transient receptor potential or TRP channels, in terms of the membrane spanning or channel core domains (Fig.1), are homologous to the voltage-gated potassium channels and other 6TM channels. However careful amino acid sequence analysis of, for example, the pore regions of these closely related channels or even for the TRP channel family members, led to the identification of sequence differences as well sequence homology. Our present understanding of the functional implications is still poor, but starts to emerge. For example, the majority of TRPM channels (TRPM1–3, 6–8) are permeable to Ca^{2+} , whereas the TRPM4 and TRPM5 channels are not. This striking difference in ion permeability was elegantly underpinned to a set of specific amino acid residues in the pore region by Nilius and colleagues (25). In the present study, we investigated two cysteine residues in the pore region; the first one is completely conserved in the TRPM subfamily and the second is present at slightly different positions (Fig.1). Substitution of either of them with alanine led to complete loss of the channel function (Fig.3). These positions were not able to tolerate any structural modifications, since substitution with serine, which only replaces the SH-group in the side chain with an OH- group, still blunted channel function (Fig.3). In contrast to C996 and C1008, mutation of a nonconserved residue (E994) gave rise to normal functional channels, indicating that the loss of function for the cysteine mutants examined here is not simply due to non-specific mutational effects.

TRPM2 are cationic channels that are permeable to Na^+ , K^+ and Ca^{2+} . Considering that C996 and C1008 are located in the pore region, mutations of pore residues could potentially alter the ion selectivity as has already been shown for the TRPM4 and TRPV6 channels (25). However whole-cell recordings show that cells expressing C996S and C1008S subunits in extracellular solutions containing Na^+ , K^+ or Ca^{2+} as the major ion species all failed to produce significant ADPR-evoked currents, in contrast to cells expressing wild type subunit (Fig.3B and C). This largely rules out that the possibility that loss of channel function was due to altered ion permeation properties of the channel.

Loss of ion channel function could however be attributed to changes in protein expression or protein trafficking, e.g., K_{ATP} channels (1, 26) and P2X receptors (2). Alternatively, it may also be due to changes in the ability of the subunits to interact to form multiple-subunit

complexes, as previously shown for cyclic nucleotide gated-channels (36), Ca^{2+} -activated potassium channels (16) and chloride channels (31). However, our Western blotting, immunostaining and biotin labelling experiments show that the mutant TRPM2 protein expression levels and membrane localisation were very similar to those of the wild type subunit (Fig.4). Furthermore, the co-immunoprecipitation experiments indicate that there were no detrimental effects on the ability of the subunits to interact as a result of mutating these cysteine residues (Fig.5A). Whole-cell current recordings from cells co-expressing mutant and wild type subunits demonstrate that co-expression of mutant subunits dramatically reduced the ADPR-evoked currents (Fig.5B). These electrophysiological results not only indicate that the wild type and mutant subunits do interact to form “heteromeric” channels, but also provide further functional evidence to support the importance of these cysteine residues in TRPM2 channel function.

TRPM2 channels, like any other ligand-gated ion channels, in the simplest model are activated in two major steps, that is, agonist binding and channel gating (conformational changes elicited by agonist binding that lead to channel opening). For TRPM2 channels, the agonist ADPR has been shown to bind to the intracellular C-terminal NUDT9-H domain (19, 27), which is physically distant from the pore region (Fig.1). Thus, it is unlikely that these cysteine residues contribute to ADPR binding. One possibility is that they may be involved in the conformational changes that occur during channel gating. However, as the cysteine SH- group is essential for channel function, an alternative interpretation is that these residues could form disulfide bonds to maintain the tertiary structure and/or conformation of the channel complex. Experiments showed that ADPR-evoked currents were not affected by application of dithiothreitol (DTT), a reducing reagent, or 2-(trimethylammonium)ethyl methanethiosulfonate, a cysteine-specific modifying reagent (data not shown). Similar observations using these modifying reagents have previously been seen for instance in the ATP-gated P2X receptors, which contain ten conserved cysteine residues on the extracellular domain (14, 15). However, subsequent studies using site-directed mutagenesis revealed that these conserved cysteines in the P2X receptor are structurally important in maintaining receptor function (5, 7). Further studies are required to determine whether these cysteine residues play a role in the channel gating and/or they contribute to the structural stability of the channel.

Co-expression of TRPM2 wild type and cysteine mutant subunits resulted in a significant suppression of the ADPR-evoked currents, and in particular the effect resulting from co-expression with C996S mutant subunit is striking. The difference in the functional effects seen between C996S and C1008S mutant subunits might be due to that C996 is completely conserved and may occupy a position that is structurally and/or functionally more critical than C1008. This study demonstrates that mutant subunits exhibit normal protein expression, membrane trafficking and subunit interactions, thus it is reasonable to assume that incorporation of wild type and mutant subunits into tetrameric channels is binomial. In such a scenario, the suppression of channel function observed with co-expression of wild type and C996S mutant subunits suggests that channels containing one or more C996S subunits are not functional. TRPM2 mRNA is abundantly expressed in the brain. Functional TRPM2 or TRPM2-like channels have also been observed in neurons and many other cells types (see introduction). A major obstacle in studying the physiological functions of TRPM2 channels is the lack of specific antagonists. The strong functional suppression by the C996S mutant subunit, regardless of the underlying mechanisms, could be feasibly explored as a useful tool to probe the physiological roles of TRPM2 channels in native cellular milieu as have been demonstrated in many previous studies (e.g., 6, 23, 29, 34, 35).

In summary, this study has combined site-directed mutagenesis, biochemical and electrophysiological approaches to provide evidence to support that two cysteine residues in

the pore region are obligatory for normal TRPM2 channel function. Both cysteine residues are present in all the other TRPM subfamily members and in particular C996 is completely conserved, and thus the present findings contribute to our evolving understanding of the structure and function of the TRPM channels.

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Figure 1. Schematic drawing of membrane topology of TRPM2 and sequence alignments of the pore regions of TRPM subfamily

Top panel, membrane topology of TRPM2 channel subunit. S1 to S6 represents the six transmembrane segments and P represents the pore loop between S5 and S6. The TRP box indicates a stretch of relatively highly conserved amino acid residues of the TRP superfamily. The NUTD9-H domain in the C-terminus is homologous to the NUTD9 proteins where the TRPM2 channel agonist ADPR is thought to bind. *Bottom panel*, amino acid residue alignments (using ClustalW) of the pore regions and part of the S5 and S6 segments of human TRPM1-8 subunits and the mouse (m) TRPM2 subunit. Residues are numbered according to the hTRPM2 subunit. Residues studied are indicated (Cysteine residues at 996 and 1008 in bold and the glutamic acid residue at 994 underlined).

Figure 2. Functional expression and properties of hTRPM2 channels

A: Representative current responses to voltage ramps from -120 mV to 80 mV in 1 s duration (*left top panel*) applied every 10 s in a cell expressing the TRPM2 channel (*left bottom panel*). Cells were held at -40 mV. The first three small current responses in grey which are almost superimposed were recorded in the cell-attached configuration. The large current responses in black were recorded after whole-cell configuration was established. The current amplitudes at membrane potentials of -80 and 80 mV are plotted over time (*right panel*). The arrow indicates the first current recorded in the whole-cell configuration. Intracellular solution contained APDR (1 mM). B: An example of the inhibition of ADPR-evoked currents by flufenamic acid (FFA, 0.5 mM for 1 min) at a membrane potential of

–80 mV recorded every 5 s. Inset shows current-voltage (I/V) relationship curves before (point 1: the 9th whole-cell recording indicated with filled circle in Fig. 2B) and after FFA inhibition (point 2: the fifth recording after addition of FFA). The arrow indicates the first current recorded in the whole-cell configuration. Inhibition was almost complete and irreversible during washing for 5 min, and was also voltage-independent. C: Summary of the mean peak currents evoked at –80 mV recorded from transfected cells with and without ADPR (1 mM) in the intracellular solution (*left panel*), before (–FFA) and after application of FFA (+FFA) from experiments shown in panel B (*middle panel*), and in extracellular solution containing 2 mM BaCl₂ without CaCl₂ (+Ba) and extracellular solution containing 2 mM CaCl₂ (+Ca) (*right panel*). The numbers of cells examined are indicated above each bar. ** $p < 0.001$ and *** $p < 0.0001$.

Figure 3. Mutations of cysteine residues result in loss of channel function

A: Currents at a membrane potential of -80 mV from cells expressing TRPM2 wild type (*left panel*) or C996S mutant channels (*right panel*) using voltage ramps as described in Fig. 2A. The arrows indicate the first current recorded in the whole-cell configuration and the grey bars show the duration of application of flufenamic acid (FFA). B: Summary of mean peak currents recorded from non-transfected HEK293 cells (HEK), and cells expressing EE-tagged TRPM2 wild type (WT), C996A, C996S, C1008A or C1008S mutant channels. *** $p < 0.0001$ indicates a significant difference in the current amplitude of wild type channels compared to mutants (C996A, C996S, C1008A or C1008S) channels and non-transfected cells. C: Mean currents from cells expressing wild type, C996S or C1008S mutant channels

in extracellular solutions containing 149 mM KCl or 110 mM CaCl₂. ** $p < 0.005$ indicates a significant difference in the current amplitude between wild type and mutant channels (C996S or C1008S). The numbers of cells examined are indicated above each bar.

Figure 4. Loss of function for mutant TRPM2 are not due to defects in expression and membrane localisation

A: Western blot analysis of whole cell lysates from non-transfected HEK293 (HEK) cells and HEK293 cells expressing EE-tagged TRPM2 wild type, C996A, C996S, C1008A and C1008S mutant subunits using an anti-EE antibody. B: Immunostaining of non-transfected HEK293 cells, and HEK293 cells expressing EE-tagged TRPM2 wild type, C996S or C1008S mutant subunits using anti-EE antibody. Note that there was no detectable immunostaining in non-transfected HEK cells and that the immunoreactivity in cells expressing TRPM2 wild type or mutant subunits were dominantly clustered around the plasma membrane. Furthermore, there was no significant difference in immunoreactivity

between wild type and mutant subunits. C. Western blot analysis of whole cell lysates (*top panel*) and biotin labelled membrane proteins (*bottom panel*) from cells expressing EE-tagged TRPM2 wild type, C996S and C1008S mutant subunits using anti-EE antibody. Similar results were observed in at least two independent experiments.

Figure 5. Mutant TRPM2 subunits when co-expressed with wild type subunit show normal subunit interaction but significantly reduced channel function

A: Western blot analysis of whole cell lysates (*top panel*) using an anti-EE antibody. Western blot analysis using anti-FLAG antibody (*middle panel*) or using anti-EE antibody (*bottom panel*) of immunoprecipitated proteins pulled down using anti-FLAG antibody. Cells were transfected with plasmids for the pairs of wild type or mutant subunits indicated above. Similar results were seen in two independent experiments. B: Current responses were recorded every 5 s from cells transfected with EE-tagged wild type (*top left*), EE-tagged wild type and C996S mutant subunits (*top right*), or EE-tagged wild type and C1008S mutant subunits (*bottom left*). Insets show the I/V relationship curves before (point 1) and

after (point 2) inhibition by FFA. The results from all the experiments are summarized as mean data (*bottom right*) and the numbers of cells examined are indicated. * $p < 0.05$, and *** $p < 0.0001$. A 1:1 plasmid ratio was used to co-express wild type and mutant subunits.