Role of the α-Kinase Domain in Transient Receptor Potential Melastatin 6 Channel and Regulation by Intracellular ATP

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Transient receptor potential melastatin 6 (TRPM6) plays an essential role in epithelial Mg\(^{2+}\) transport. TRPM6 and its closest homologue, TRPM7, both combine a cation channel with an α-kinase domain. However, the role of this α-kinase domain in TRPM6 channel activity remains elusive. The aim of this study was to investigate the regulation of TRPM6 channel activity by intracellular ATP and the involvement of its α-kinase domain. We demonstrated that intracellular Na- and Mg-ATP decreased TRPM6 current in HEK293 cells heterogeneously expressing the channel, whereas Na-CTP or Na-GTP had no effect on channel activity. Whole cell recordings in TRPM6-expressing HEK293 cells showed that deletion of the α-kinase domain prevented the inhibitory effect of intracellular ATP without abrogating channel activity. Mutation of the conserved putative ATP-binding motif GXG(A)XXG (G1955D) in the α-kinase domain of TRPM6 inhibited the ATP action, whereas this effect remained preserved in the TRPM6 phosphotransferase-deficient mutant K1804R. Mutation of the TRPM6 autophosphorylation site, Thr\(^{1851}\), into either an alanine or an aspartate, resulted in functional channels that could still be inhibited by ATP. In conclusion, intracellular ATP regulates TRPM6 channel activity via its α-kinase domain independently of α-kinase activity.

Within the transient receptor potential family of cation channels, three members from the Melastatin subgroup, TRPM6, TRPM7, and TRPM2, display unique primary structures known as “chanzymes,” i.e. fusion of an ion channel pore-forming region with an enzymatic domain (1–5). Remarkably, the proteins TRPM6 and TRPM7 share 52% sequence identity and play a crucial role in Mg\(^{2+}\) homeostasis (6, 7). Mutations in the TRPM6 gene are responsible for the autosomal recessive form of familiar hypomagnesemia with secondary hypocalcemia (8, 9), whereas a missense mutation in TRPM7 is responsible for the pathogenesis of Guamanian neurodegenerative disorders (10). In addition, the lethal phenotype caused by inactivation of the TRPM7 gene in lymphocytes can be rescued by extracellular Mg\(^{2+}\) supplementation (1, 11).

Although the physiological activation mechanism of TRPM6 remains unknown, there is evidence of a constitutive activity in heterologous expression systems. TRPM6 displays a nonselective cation current conducted by divalent ions inwardly and monovalent ions outwardly and exhibits a steep outwardly rectifying current-voltage relation (12). A recent study reported that epidermal growth factor hormone acts as an autocrine/paracrine magnesiotropic hormone, specifically stimulating TRPM6 current via engagement of its receptor at the basolateral membrane of distal convoluted tubule cells (13). Previous studies showed that both TRPM6 and TRPM7 currents are inhibited by millimolar concentrations of intracellular Mg\(^{2+}\) (1, 11, 12). In rat basophilic leukemia and Jurkat T cells, a native conductance with properties similar to cells heterogeneously expressing TRPM7 was described and termed magnesium nucleotide-dependent metal cation channel (14) or Mg\(^{2+}\)-inhibited cation channel (15, 16). Subsequent studies reported critical amino acids in the pore regions of TRPM7 (Glu\(^{1047}\)/Glu\(^{1052}\)) and TRPM6 (Glu\(^{1024}\)/Glu\(^{1029}\)) that play a role in Mg\(^{2+}\) and pH sensitivity (17). The functional α-kinase domain at the carboxyl terminus of TRPM7 has been structurally characterized by x-ray crystallography (4). In contrast to the classical kinases, the α-kinase domain displays unique features including a zinc finger domain as well as a region involved in the catalysis of ATP-triggered reactions (18). Despite this information, the role of intracellular ATP on the regulation of channel activity remains controversial (19–22). The current consensus is that ATP acts as an intracellular Mg\(^{2+}\) chelator, which decreases the concentration of intracellular free Mg\(^{2+}\), resulting in increased TRPM7-mediated conductance (19–22). These observations were challenged by a study demonstrating that both Mg\(^{2+}\) and Mg\(^{2+}\)-nucleotides act in synergy to inhibit TRPM7 activity via two distinct binding sites located within the carboxyl terminus (22). Another debated aspect concerns the role of the α-kinase in modulation of channel activity. Some studies reported that phosphotransferase-deficient TRPM7 mutants showed a reduced sensitivity for intracellular Mg\(^{2+}\) (11), exhibited a reduced current amplitude upon G protein-coupled receptor stimulation (23), or were even not functional (2), whereas others found that TRPM7 lacking the α-kinase...
domain displayed similar Mg$^{2+}$ sensitivity compared with the wild-type channel (24). In contrast to the TRPM7 $\alpha$-kinase domain little is known about the TRPM6 $\alpha$-kinase domain. Recently, TRPM6 activity has been shown to be controlled by RACK1 via its interaction with the $\alpha$-kinase (25). An additional study reported that the $\alpha$-kinase of TRPM6 is capable of cross-phosphorylation of TRPM7 but not vice versa, underlying the functional nonredundancy of these two chanzymes (26). In this line, Li et al. (27) demonstrated that TRPM6, TRPM7, and TRPM6/7 are three distinct ion channels that exhibit different functional characteristics, thereby showing unequivocally that TRPM6 can form by itself functional channels without TRPM7 co-expression. Although the previously reported data suggested that the ATP effect on TRPM7 is independent of $\alpha$-kinase activity, there are no potential domains identified outside of the $\alpha$-kinase domain mediating a direct interaction with ATP. Notably, renal distal convoluted tubule cells contain a large number of mitochondria per unit length of any cell along the nephron, underling a dynamic capacity of intracellular ATP (28). Given the predominant expression pattern of TRPM6 in the distal convoluted tubule (12), the molecular mechanism of ATP-regulated TRPM6 channel activity was investigated.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (Bio Whittaker-Europe, Verviers, Belgium) containing 10% (v/v) fetal calf serum, 2 mM Dulbecco’s modified Eagle’s medium (Bio Whittaker-Europe, Verviers, Belgium) was used to estimate the free Mg$^{2+}$ concentration. The osmolality of the solutions was adjusted to $\pm$310 mOsm/kg with mannitol. All of the chemicals were purchased from Sigma.

**Immunoblotting**—72 h after transfection, HEK293 cells were lysed, and protein samples were denatured by incubation for 30 min at 37 °C in Laemmlli buffer and then subjected to SDS-PAGE (50 $\mu$g/lane). Immunoblots were incubated with the mouse anti-HA (Sigma) antibody. Subsequently, the blots were incubated with sheep horseradish peroxidase-conjugated antimouse IgG (Sigma) and then visualized using an enhanced chemiluminescence system.

**In Vitro Phosphorylation Assays**—72 h after transfection, HEK293 cells were lysed for 1 h on ice in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5 adjusted by NaOH, 1% (v/v) Nonidet P-40 including the protease inhibitors leupeptin (0.01 mg/ml), pepstatin (0.05 mg/ml), and phenylmethylsulfonyl fluoride (1 mM)). After centrifugation of the lysates, supernatants were incubated with anti-HA antibody immobilized on protein A-agarose beads (Kem-En-Tec A/S, Copenhagen, Denmark) for 3 h at room temperature. Following three washing steps with lysis buffer, the beads were incubated in a total volume of 30 $\mu$l of kinase reaction buffer (50 mM HEPES, pH 7.4 adjusted by KOH, 4 mM MnCl$_2$, 0.5 mM CaCl$_2$, 100 $\mu$M ATP) and 2 $\mu$Ci of [γ-32P]ATP for 30 min at 30 °C. Following 30 min of TRPM6 phosphorylation the reaction was terminated by three washing steps with phosphorylation washing buffer (50 mM HEPES, pH 7.4 adjusted by KOH, 4 mM MnCl$_2$, 0.5 mM CaCl$_2$). Phosphorylation was analyzed after gel electrophoresis by autoradiography.
Expression and Purification of GST Fusion Proteins—GST-fused proteins were expressed according to the manufacturer's protocol (Amersham Biosciences). To purify the GST fusion proteins, the cell pellets were lysed in cold lysis buffer (50 mM Tris, pH 7.4 adjusted by NaOH, 120 mM NaCl, 0.5% (v/v) Nonidet P-40, 1 mM EDTA, 0.5 mg/ml lysozyme including the protease inhibitors leupeptin (0.01 mg/ml), pepstatin (0.05 mg/ml), and phenylmethylsulfonyl fluoride (1 mM)) for 40 min and subsequently centrifuged for 30 min at 4 °C. The supernatant was added directly to glutathione-Sepharose 4B beads (Amersham Biosciences) for purification. After 2 h of incubation at room temperature, the beads were washed extensively with lysis buffer. The bound proteins were eluted with SDS-PAGE loading buffer, separated on SDS-polyacrylamide gel, and detected by Coomassie staining.

**ATP Binding Assay**—GST and GST-kinase fusion proteins were purified as described above, and subsequently ~100 ng was incubated with 2 μCi of [γ-32P]ATP in 50 μl of ATP binding buffer (45 mM HEPES (13), 10 mM EDTA, 0.9 mM EGTA, 0.14 mM KCl, 9% v/v glycerol, 0.018% v/v Nonidet P-40, and 0.12 mg of bovine serum albumin/ml) for 20 min at 37 °C. After three wash steps with ATP binding buffer in the absence of [γ-32P]ATP, the samples were analyzed by scintillation counting.

**Sequence Analysis and Structure Modeling**—A structural model of the TRPM6 α-kinase domain was built based on the crystal structure of the TRPM7 α-kinase domain (4) (Protein Data Bank code 1IA9 (AMP-PNP complex)) using the WHAT IF/Yasara Twinset (31). The standard parameter settings were used. The sequence alignment and all other relevant modeling information is available online (supplemental Figs. S1 and S2).

**Data Analysis**—In all experiments, the data are expressed as the means ± S.E. Overall statistical significance was determined by analysis of variance. In case of significance, differences between the means of two groups were analyzed by unpaired t test. p < 0.05 was considered significant. Statistical analysis was performed using the SPSS software (SPSS Inc., Chicago, IL).

**RESULTS**

**Effect of Intracellular ATP on TRPM6 Channel Activity**—To investigate the functional properties of TRPM6, electrophysiological measurements were performed on transiently transfected HEK293 cells expressing HA-TRPM6. These cells developed large whole cell currents that reached their maximal amplitude (260 ± 30 and −10 ± 1.5 pA/pF at +80 and −80 mV, respectively) within a 200-s time period. These currents were not observed in mock transfected cells (Fig. 1A). TRPM6 displayed a characteristic outwardly rectifying current-voltage relation (Fig. 1B). Moreover, as already described (12), outwardly rectifying currents were also observed in mock transfected HEK293 cells dialyzed with a Mg2+-free pipette solution (containing 10 mM EDTA). However, when compared with TRPM6-transfected cells, these currents developed over a longer time period and reached maximal amplitudes that are ~10 times smaller (Fig. 1A). Next, in the presence of 10 mM Na-ATP and no added Mg2+ in the patch pipette solution, TRPM6 currents activated slowly and reached its maximal amplitude after 200 s (Fig. 1, C and D, 102 ± 25 and −5.9 ± 1.5 pA/pF at +80 and −80 mV, respectively). This maximal amplitude was 2.5-fold reduced in comparison with the wild-type current. Perfusion of higher Na-ATP concentrations (12 mM) abrogated its development (Fig. 1, C and D). Because ATP is well known to sequester free intracellular Mg2+ (32), it was essential to determine whether the inhibitory Na-ATP effect results from Mg2+ buffering or from direct inhibition of channel activity. Considering that TRPM6 is inhibited by increased intracellular Mg2+ levels (12), the inhibitory effect of ATP cannot be due to its Mg2+ chelator property. However, to clarify this point, we performed the experiments in the presence of 10 mM EDTA, a strong Mg2+ chelator (free [Mg2+]p ~0 mM as calculated with Calcu program). Importantly, the addition of Na-ATP (2 mM) in the presence of 10 mM EDTA significantly reduced the TRPM6-mediated current at both +80 and −80 mV (Fig. 1, E and F, −46 ± 6% and −53 ± 2%, respectively, p < 0.05, n = 17–22 cells). Furthermore, replacing EDTA with HEDTA in the patch pipette solution did not affect TRPM6 activity (supplemental Fig. S3). As summarized in Fig. 1G, after breaking the membrane (0 s), TRPM6 current amplitude was increased in the presence of EDTA compared with Na-ATP. In the presence of 10 mM EDTA, the addition of either 2 mM Na-ATP 10 mM Na-ATP or 12 mM Na-ATP in the patch pipette reduced TRPM6 current development in comparison with recordings with only 10 mM EDTA. These data indicate that free ATP inhibits TRPM6 activity independently of the intracellular Mg2+ concentration. To further confirm this hypothesis, we substituted Na-ATP with Mg-ATP, a physiological form of ATP in the patch pipette solution. In these experiments the intracellular Mg2+ concentration was clamped at micromolar concentrations with 10 mM EDTA. Our data showed that Na-ATP and Mg-ATP inhibit TRPM6 currents in a similar dose-dependent manner with a comparable IC50 of ~1.3 mM (Fig. 1H).

**Specificity and Reversibility of the ATP Effect on TRPM6**—To evaluate the specificity of intracellular ATP on TRPM6 activity, TRPM6-transfected HEK293 cells were perfused with Na+-nucleotide triphosphates. Neither Na-GTP nor Na-CTP was able to affect the TRPM6 current compared with the nucleotide-free controls, whereas Na-ATP inhibited the current at +80 and −80 mV (Fig. 2). Na+ and/or nucleotides triphosphates did not affect the current in mock transfected cells.

**Relevance of the α-Kinase Domain for TRPM6 Channel Activity**—To investigate the role of α-kinase domain in the inhibitory ATP effect, the α-kinase domain was truncated by introducing a stop codon at position 1749 (Δkinase; see Fig. 4A). This Δkinase mutant was subsequently analyzed for its auto-phosphorylation activity. *In vitro* phosphorylation experiments revealed that truncation of the α-kinase domain abolished kinase activity in contrast to the wild-type channel (Fig. 3A, top panel). Immunoblot analysis indicated equal expression of both TRPM6 and Δkinase (Fig. 3A, lower panel). Next, the Δkinase mutant was functionally characterized and its sensitivity to Na-ATP was studied. HEK293 cells expressing Δkinase displayed currents with lower amplitude compared with the wild-type channel at both +80 and −80 mV (55 ± 14% of the wild type remained, p < 0.05, n = 16–19 cells) but with
similar activation kinetics (Fig. 3, B and C). The current-voltage relation of Δkinase is comparable with wild-type TRPM6 (Figs. 1B and 3F). Na-ATP (2 mM) did not affect either the current amplitude at 200 s (Fig. 3, D and E) or the current-voltage relation of the Δkinase mutant (Fig. 3F), indicating the importance of the α-kinase in the inhibitory ATP effect. Subsequently, the role of this domain was studied by site-directed mutagenesis (Fig. 4A). First, based on sequence homology, the mutations rendering TRPM7 phosphotransferase-deficient (K1648A and D1775A (11)) were introduced in TRPM6 (positions 1804 and 1933 for K1804R and D1933A, respectively). Second, the Thr1851 that has been recently identified as an auto-phosphorylated amino acid residue within the α-kinase (25) was either point-mutated into an alanine, resulting in a putative non-phosphorylated mutant (T1851A) or into an aspartate, mimicking a constitutive auto-phosphorylated mutant (T1851D). Using in vitro phosphorylation experiments, the phosphotransferase-deficient mutants, D1933A and K1804R showed an abolished α-kinase activity in contrast to the wild-type channel (Fig. 4B, top panel). Furthermore, the T1851A and T1851D mutants exhibited reduced auto-phosphorylation in comparison with the wild-type channel (Fig. 4B, top panel). Immunoblot analysis indicated comparable expression of the (mutated) TRPM6 proteins (Fig. 4B, bottom panels). Next, all of the mutants were functionally characterized in whole cell recordings. HEK293 cells expressing the phosphotransferase-deficient mutant K1804R exhibited current amplitudes similar to the wild-type channel, whereas the phosphotransferase-deficient D1933A mutant had no activity (Fig. 4, C and D). In addition, both T1851A (nonphosphorylated) and T1851D (constitutively phosphorylated) mutants resulted in active channels with comparable current amplitudes as wild-type TRPM6 (Fig. 4, C and D). Subsequently, Na-ATP was tested on the functional TRPM6 mutants. Na-ATP perfusion reduced both inward and outward K1804R, T1851A, and T1851D-mediated currents (37 ± 4, 36 ± 7, and 55 ± 6% of the current remained at +80 mV, respectively; Fig. 5, A–F). No effect of Na-ATP was observed on the current-voltage relations (Fig. 5, G–I).
Role of GXG(A)XXG Loop in ATP-mediated Channel Regulation—To elucidate the molecular mechanism of the ATP effect on TRPM6 channel activity, the three-dimensional structure of the TRPM6 α-kinase domain was modeled and analyzed using the WHAT IF/Yasara Twinset. Detailed analysis of the TRPM6 α-kinase model revealed an ATP-binding pocket, similar to the TRPM7 template, that is flanked by the conserved GXG(A)XXG loop (4) (Fig. 6A). To investigate the role of this domain, the glycine at position 1955 was mutated into an aspartate (G1955D), and in vitro phosphorylation assays revealed that this mutation abolished TRPM6 auto-phosphorylation (Fig. 6B, upper panel), whereas immunoblot analysis indicated equal expression of the (mutated) TRPM6 proteins (Fig. 6B, lower panel). Subsequent Patch clamp analysis revealed that the G1955D mutant is a functional channel with similar current, amplitude, and current-voltage relation as the wild-type channel (compare Figs. 1B and 6D and Figs. 1B and 6E, respectively). Interestingly, G1955D mutation prevented the inhibitory ATP effect without affecting the current-voltage relation (Fig. 6, C and D). Next, in vitro ATP binding studies were performed to address whether ATP binding is affected in the G1955D mutant. As demonstrated in Fig. 7A, ATP is significantly bound by the wild-type α-kinase domain in comparison with GST alone. However, ATP binding was not disturbed in the G1955D mutant excluding this amino acid from the ATP-binding pocket. Equal expression input of the purified GST fusion proteins in the ATP binding assay was confirmed by Coomassie staining on SDS/PAGE gel (Fig. 7B). To further investigate why the G1955D mutant was not inhibited by ATP, the three-dimensional model of the α-kinase domain was analyzed by Yasara Twinset. As shown in Fig. 7C, the Gly1955 or Asp1955 residues in the conserved GXG(A)XXG motif are localized far away from the ATP molecule within the ATP-binding pocket, suggesting that the G1955D mutation may not have a direct influence on ATP binding. Based on the three-dimensional structure of the crystallized TRPM7 α-kinase domain, which was used as a template to model the TRPM6 α-kinase domain structure, and the B-factor analysis we found that the conserved GXG(A)XXG loop in the α-kinase domain is quite flexible (4). The original glycine (Gly1955) residue is highly flexible in contrast to the aspartic residue, known to confer rigidity to peptides (33).

DISCUSSION

The present study provides molecular insights into the inhibitory effect of Na-ATP on TRPM6 channel activity. The intracellular ATP effect depends on the Gly1955 residue within the TRPM6 α-kinase domain, whereas TRPM6 α-kinase activity is not required. This conclusion is based on the following experimental findings. First, TRPM6 activity was inhibited by Na- and Mg-ATP in HEK293 cells heterogeneously expressing the channel. Second, this inhibition was specific for ATP because the experiments were conducted in the presence of strong Mg2+ chelators (EDTA and HEDTA), and neither GTP nor CTP mimicked the ATP action. Third, the inhibitory ATP...
TRPM6 Regulation by ATP

A

B

C

D

FIGURE 4. Functional analysis of TRPM6 α-kinase domain mutants. A, sequence of TRPM6 α-kinase domain is boxed in black. Residues targeted for mutational analysis and the truncation site of the α-kinase domain are designated by solid black circles (L1749X stop codon corresponding to Δ kinase, K1804R, T1851A/D, D1933A, and G1955D, respectively). B, TRPM6, K1804R, D1933A, T1851D, and T1851A mutants were immunoprecipitated, subjected to in vitro protein kinase assay, and analyzed by autoradiography (top). The expression of all constructs was verified by immunoblotting (bottom). Representative results of three independent experiments are shown. C and D, histogram summarizing the averaged time course of current density ± S.E. of n = 9–12 cells, at +80 mV (C) and −80 mV (D), 200 s after whole cell establishment, from HEK293 cells transfected with wild-type TRPM6, K1804R, D1933A, T1851A, and T1851D mutants, when dialyzed with standard pipette solution. The asterisk indicates p < 0.05 compared with TRPM6. The currents were measured as described in the legend of Fig. 1A.

Regulation of TRPM6 Activity by Intracellular ATP—Our data demonstrated that intracellular ATP, but not GTP or CTP, specifically inhibited TRPM6 channel activity. Physiological concentrations of intracellular Mg2+ are in the range of 0.5–1.0 mM, whereas intracellular ATP is in the range of 1–6 mM (34–36). In the presence of 10 mM Na-ATP, which buffers intracellular free Mg2+, TRPM6 current amplitude is significantly reduced. TRPM6 channel activity is abolished when the Na-ATP concentration is raised to 12 mM. The physiological relevance of this current inhibition is supported by the fact that it occurs at a membrane potential of −80 mV. We assume that the reduced current amplitude, which can be measured immediately after breaking in the membrane at +80 mV, might be due to an unstable state in the presence of such high ATP concentration. A previous study conducted in rat basophilic leukemia cells showed that Mg-ATP reduced the Mg2+-inhibited cation channel current in the presence of a weak Mg2+ chelator but not by a strong Mg2+ chelator like HEDTA (19). However, we excluded the involvement of the chelator by measuring a similar current inhibition in the situation that Na-ATP is perfused with strong chelators such as EDTA or HEDTA. Our results support that both Na- and Mg-ATP exert comparable inhibitory effects on TRPM6 (IC50 of ~1.3 mM). In contrast to TRPM7 where Mg-GTP and Mg-CTP were found to suppress TRPM7 currents (1, 22), TRPM6 is not modulated by GTP and CTP. In addition, cytosolic GTP levels are at least five times lower than ATP levels, which suggests that ATP would rather act as a physiological regulator of TRPM6.

Role of the α-Kinase Domain in TRPM6 Channel Activity—The α-kinase domain present in TRPM6 and TRPM7 is distinct in structure compared with the superfamily of eukaryotic protein kinases and resembles the elongation factor-2 kinase and other α-kinases (3). The role of this enzymatic domain on channel activity remains controversial. Based on analysis of the protein domain prediction program SMART, we identified that the α-kinase domain in human TRPM6 is located within the carboxyl-terminal region between amino acids 1749 and 2022. In this respect, the TRPM6 α-kinase region is slightly shorter than the considered domain in a previous TRPM7 study (2). Our data demonstrate that despite preventing the auto-phosphorylation activity, truncation of the α-kinase domain from position 1749 does not abolish TRPM6 channel activity. In addition, the phosphotransferase-deficient (K1804R) and the autophosphorylation-deficient (T1851A) mutations result in channels with comparable current amplitudes after 200 s as wild-type TRPM6. These observations suggest that the activity of the TRPM6 α-kinase

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domain is not necessary for channel activation. However, the Δkinase mutant current is reduced in comparison with the wild-type channel despite equal expression levels as previously demonstrated (25). In this line, it has been reported that phosphotransferase-deficient TRPM7 mutants show a reduced sensitivity for intracellular Mg$^{2+}$ (11), exhibit a reduced current amplitude upon G protein-coupled receptor stimulation (23), or are not functional (2). In addition, one study showed that the TRPM6 α-kinase-deleted mutant from position 1725 is indeed deficient for auto-phosphorylation and cross-phosphorylation of TRPM7 (26). It was postulated that TRPM6 requires assembly with TRPM7 to form functional channel complexes at the plasma membrane (26, 37). However, Li et al. (27) demonstrated that TRPM6, TRPM7, and TRPM6/7 are three distinct ion channels that exhibit different functional characteristics.

We showed that upon TRPM6 α-kinase deletion, the ATP effect is prevented, whereas it has been demonstrated that TRPM7 lacking the α-kinase domain is still sensitive to Mg$^{2+}$-ATP inhibition (22). In line with previous studies, TRPM6 and TRPM7 display distinct functional properties. TRPM6 can phosphorylate TRPM7, but not vice versa. At the transcription level, TRPM6 expression is regulated by dietary Mg$^{2+}$ content and estrogens, whereas TRPM7 mRNA abundance remains unaltered under these conditions (38). At the functional level, epidermal growth factor can specifically stimulate TRPM6 but not TRPM7 channel activity (13). Moreover, compared with TRPM7, TRPM6 is less sensitive to extracellular pH and can be activated by 2-aminoethoxydiphenyl borate (12, 17, 27). Taken together these data indicated that the molecular regulation of the two channels is divergent. Here our data demonstrate that the inhibitory effect of ATP requires the TRPM6 α-kinase domain, which further substantiates that TRPM6 and TRPM7 are channels with distinct properties. Taken together, our data show that ATP exerts its inhibitory effect independently of Mg$^{2+}$ chelation, thereby suggesting a direct modulation of TRPM6 channel activity.

Molecular Mechanism of ATP-mediated TRPM6 Inhibition—

Based on sequence similarities with the “kinase-dead” TRPM7 K1648R mutant (4, 11, 22), the Lys$^{1804}$ in TRPM6 has been mutated into an arginine. In vitro phosphorylation experiments indeed confirmed that the TRPM6-K1804R mutant is phosphotransferase-deficient. Importantly, this mutant can still be inhibited by intracellular Na-ATP. This excludes the involvement of α-kinase activity in the action of ATP. A recent report proposed that the nucleotide-dependent inhibition of TRPM7 is abolished by inactivating the α-kinase (22). Moreover, in contrast to previous reports, mutation of Gly$^{1999}$ (to aspartate) in TRPM6 (a residue equivalent to the Gly$^{1999}$ in TRPM7 studied by Schmitz et al. (11) and Matsushita et al. (24)) resulted in a functional channel. Mutation of Glu$^{1799}$ in TRPM7 resulted in a nonfunctional channel and may involve incorrect folding or disturbed trafficking. However, the plasma membrane localization of this later mutant has not been addressed. Furthermore, the Thr$^{1851}$ residue located within the TRPM6 α-kinase domain was recently identified as an auto-phosphorylation site (25). Mutation of Thr$^{1851}$ into an alanine or an aspartate resulted in functional channels that exhibited a reduced but not abolished auto-phosphorylation activity. This latter finding could be explained by the presence...
of other serine and threonine residues located in and upstream of the TRPM6 α-kinase domain (NP_060132). In this respect, two major auto-phosphorylated residues, Ser1511 and Ser1567, have been identified in TRPM7 (24). Interestingly, perfusion of intracellular ATP affected both T1851A and T1851D currents, which further supports that the ATP-mediated inhibition of TRPM6 is not dependent on the α-kinase activity. The GXG(A)XXG loop is highly conserved throughout many ATP-binding protein families (30). The TRPM6 α-kinase domain also contains this loop, and our Patch clamp analysis revealed that mutation of Gly1955 to Asp1955 eradicated the inhibitory ATP effect on channel activity. However, the in vitro ATP binding assay showed that the Gly1955 mutation has no influence on the ATP binding effect. In this line, three-dimensional structure analysis also suggested that Gly1955 may not be directly involved in ATP binding. In the absence of ATP, the TRPM7 α-kinase structure shows a disordered GXG(A)XXG loop (4), corroborating the flexibility of this loop. In this line, the B-factor analysis of the corresponding GXG(A)XXG loop in the α-kinase of TRPM7 suggests high conformational flexibility. When the Gly1955 is mutated to Asp1955, which contains much lower conformational flexibility than glycine, the GXG(A)XXG loop becomes more rigid. It seems likely that the GXG(A)XXG loop is involved in transferring the signal generated by ATP binding to the other parts of TRPM6. Thus, the reduced mobility of this loop does not diminish ATP binding but more likely may cause a less optimal structure for transfer or communication of the ATP-binding signal to other parts of TRPM6 protein.

In conclusion, our results unveiled that intracellular Na-ATP, but not Na-GTP and Na-CTP, specifically inhibits TRPM6 channel activity. Moreover, the α-kinase domain but not kinase activity is required for this regulatory effect of ATP. The inhibitory effect of ATP on TRPM6 current is abolished by a single mutation in the GXG(A)XXG loop located within the

FIGURE 6. Role of G1955D mutation within the GXG(A)XXG loop in the ATP effect. A, ATP-binding pocket in the modeled three-dimensional model of the TRPM6 α-kinase domain. The ATP molecule and the conserved GXG(A)XXG loop are indicated by arrows. The ATP molecule is depicted, and the GXG(A)XXG loop is colored in yellow. B, TRPM6 and G1955D mutants were immunoprecipitated, subjected to in vitro protein kinase assay, and analyzed by autoradiography (top panel). The expression of all constructs was verified by immunoblotting (bottom panel). Representative results of three independent experiments are shown. C and D, application of 2 mM ATP in the patch pipette reduced G1955D-mediated current (black triangles) in comparison with the control conditions (0 mM [ATP], open triangles) at +80 (C) and −80 mV (D). The data represent the means ± S.E. of n = 19–21 cells. E, current-voltage relation from HEK293 cells transfected with G1955D in presence or absence of 2 mM ATP in the patch pipette as indicated. The currents were measured as described in the legend of Fig. 1A.

FIGURE 7. Functional analysis of the G1955D mutant. A, in vitro ATP binding assay of GST, GST-TRPM6 α-kinase domain (TRPM6), and GST-G1955D α-kinase mutant (G1955D). The asterisks indicate p < 0.05 compared with GST, n = 3. B, Coomassie staining of the purified GST fusion proteins on SDS/PAGE gel. C, comparison of the putative three-dimensional model of TRPM6 and G1955D α-kinase domain. ATP is depicted and indicated by an arrow, and the GXG(A)XXG loop is colored in green. The Gly1955 and Asp1955 positions are indicated by circles, and their flexibility is indicated. The ATP molecule is indicated by an arrow.

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α-kinase domain. These findings highlight that, in addition to its phosphorylation activity, the α-kinase domain itself plays an important role in TRPM6 regulation.

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