Insight into the molecular regulation of the epithelial magnesium channel TRPM6
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Purpose of review
Recent studies have greatly increased our knowledge concerning the molecular mechanisms of renal magnesium handling. This review highlights the functional features of the newly identified transient receptor potential channel melastatin subtype 6 (TRPM6), which forms the gatekeeper of active magnesium reabsorption in the kidney.

Recent findings
TRPM6 confines a magnesium permeable channel of which the expression is regulated by multiple factors, including dietary magnesium, magnesiotropic hormones and drugs. TRPM6 channel activity is modulated by intracellular magnesium and pH. A recently identified point mutation in the pro-epidermal growth factor (EGF) gene, causing isolated recessive inherited renal hypomagnesemia, implicated EGF as a magnesiotropic hormone regulating TRPM6 activity. Furthermore, receptor for activated C-kinase (RACK1) was identified as the first associated protein of the TRPM6 α-kinase domain, which acts as a dynamic switch controlling TRPM6 activity in an autophosphorylation-dependent manner. Of note, the fused α-kinase domain functions as a sensor of the intracellular magnesium concentration and plays a feedback role in controlling TRPM6-mediated magnesium influx, preventing magnesium overload during epithelial magnesium transport.

Summary
The diverse molecular regulation of TRPM6 by magnesiotropic hormones, intracellular factors and its fused α-kinase domain disclosed novel regulatory mechanisms of active magnesium reabsorption.

Keywords
α-kinase domain, epidermal growth factor, estrogen, Mg2+ homeostasis, RACK1, TRPM6

Introduction
Whole body magnesium homeostasis is of crucial importance for various pathophysiologic and physiological processes, including hypertension, neuromuscular excitability, metabolic syndrome, neuroprotection and immune system [1–6]. The magnesium balance is tightly controlled by the concerted action of intestinal absorption, exchange of magnesium from bone and renal magnesium reabsorption. In the kidney, the majority of magnesium reabsorption is localized in the thick ascending limb of Henlé’s loop, while the reabsorption in the distal convoluted tubule (DCT) defines the final urinary magnesium excretion. Importantly, magnesium transport in this latter segment occurs in an active and transcellular manner [7–10]. The molecular details and regulation of this pathway remain incompletely understood.

TRPM6, the gatekeeper of active renal magnesium reabsorption
The transient receptor potential melastatin subtype 6 (TRPM6) was recently identified as a pivotal component in active magnesium absorption and reabsorption. Mutation of this gene causes hypomagnesemia with secondary hypocalcemia (HSH) as demonstrated by two groups independently using a positional cloning approach [11,12]. TRPM6 belongs to the TRP subfamily of the superfamily of transient receptor potential (TRP) channels and is so far the only known channel directly mediating active transepithelial magnesium transport [11,12]. TRPM6 is abundantly expressed in colon, lung and kidney [13]. Further immunohistochemical analysis demonstrated that TRPM6 is predominantly localized along the luminal membrane of DCT cells, supporting an important role for this channel in active magnesium reabsorption [12–14].
TRPM6 contains six putative transmembrane spanning domains with a pore region between the fifth and sixth segment, and large intracellular amino (N) and carboxyl (C) termini. TRPM6 acts as a divalent cation channel which displays strong outward rectification measured by patch clamp analysis. The TRPM6 activity is tightly regulated by intracellular magnesium levels and is blocked in a voltage-dependent manner by ruthenium red, a highly charged polycationic reagent [14]. TRPM6 may form a functional homotetrameric channel or a heterotetramer with its closest homologue, TRPM7, a crucial protein for cellular magnesium homeostasis. There is controversy, however, about the role of TRPM7 on the functional expression of TRPM6, which needs to be further clarified [15–18]. TRPM6 exhibits a unique unitary conductance that is approximately two-fold greater than that of TRPM7 and the TRPM6/7 heterotetramer [16]. Of note, TRPM6 has an approximately five-fold higher affinity for magnesium than for calcium, while all other known calcium-permeable channels generally display a 10–1000 times lower affinity for magnesium than for calcium [14]. Further detailed functional analysis of the putative pore region highlights amino acid residues E1024, E1029, I1030 and D1031 as important determinants of cation permeation through TRPM6 [19,20]. Together, the combined properties of selective magnesium permeability, impaired magnesium homeostasis in patients with HSH and the specific epithelial expression pattern of this channel emphasize the important role of TRPM6 in magnesium absorption.

**Regulation of TRPM6 activity**

Potentially, the regulation of TRPM6 channel activity can be controlled at discrete processing levels, including transcription, translation, posttranslational modification, trafficking to the plasma membrane, channel gating and ultimately protein degradation. Although it remains elusive whether these regulatory processes all apply to TRPM6, we will herein summarize the recent discoveries of the molecular mechanisms controlling TRPM6 activity including the role of novel magnesiotropic hormones, dietary magnesium, several intracellular factors and the fused α-kinase.

**Regulation by magnesiotropic hormones**

It was previously shown that estrogen influences whole body magnesium balance, which may explain the hypermagnesiuria in postmenopausal women [21–24]. Recently, Groenestege et al. [13] demonstrated that the renal TRPM6 mRNA level in ovariectomized rats is significantly reduced, whereas supplementation with 17β-estradiol normalizes TRPM6 mRNA levels in these rats. Of note, the renal mRNA abundance of TRPM7 remained unaltered under these conditions [13]. This finding provides insight into the molecular mechanism of the estrogen deficiency-induced hypermagnesiuria. Furthermore, accumulating evidence suggests that in addition to its effect on the transcriptional pathway, estrogen has the ability to activate rapid signaling cascades via its plasma membrane receptor [25–28]. Therefore, acute effects of estrogen on TRPM6 channel activity need to be further investigated.

A recent study [29**] identified epidermal growth factor (EGF) as a magnesiotropic hormone directly stimulating TRPM6 activity. Genetic analysis and a positional cloning strategy revealed that a point mutation in the pro-EGF gene causes a rare inherited autosomal recessive form of renal hypomagnesemia (IRH). The authors suggest that this mutation in pro-EGF impairs the release of this hormone to the basolateral membrane in the DCT, disturbing the stimulatory effect of EGF on TRPM6 activity and causing renal magnesium wasting [29**]. Interestingly, it has been shown that magnesium wasting occurs in patients with colorectal cancer who are treated with EGF receptor (EGFR)-targeted antibodies [30]. Cetuximab, an EGFR-specific monoclonal antibody that prevents receptor stimulation by EGF, has been widely used as chemotherapy for patients with colorectal cancer [31–33]. Serum magnesium levels, however, gradually decreased during cetuximab treatment [29**]. These findings support a potential role for EGFR-signaling in regulating magnesium reabsorption [34]. Further investigations demonstrated that EGF can act as an autocrine/paracrine magnesiotropic hormone, specifically increasing TRPM6 activity via engagement of its receptor on the basolateral membrane of DCT cells. This effect is specific for TRPM6, since the stimulatory effect of EGF is not observed on TRPM7 [29**]. These findings provide the first insight into the molecular regulation of TRPM6 by extracellular EGF. Moreover, it demonstrates the molecular basis for the side effect of cetuximab treatment on colorectal cancer and indicates TRPM6 as a potential pharmacological target during cetuximab therapy. It has been shown that EGF stimulation facilitates the insertion of TRPC4 and TRPC5 channels into the plasma membrane [35,36]. Therefore, it would be of interest to investigate whether the stimulatory effect of EGF on TRPM6 utilizes the same mechanism.

In addition to estrogen and EGF, other hormones, including insulin, isoproterenol, arachidonic acid and prostaglandins, have been suggested to influence the magnesium homeostasis [37]. Therefore, studies addressing the role of these hormones on TRPM6 regulation may reveal new avenues for understanding active magnesium absorption and reabsorption.

**Regulation by dietary magnesium**

To maintain whole body magnesium balance, magnesium excretion is strongly regulated by dietary magnesium. Previous studies indicated that magnesium
restriction results in renal magnesium conservation, whereas a magnesium-enriched diet increases urinary magnesium excretion [13]. As the gatekeeper of active magnesium reabsorption, the expression level of TRPM6 is also tightly regulated. Interestingly, magnesium restriction significantly upregulated renal TRPM6 mRNA levels, whereas a magnesium-enriched diet increased TRPM6 mRNA expression in colon as demonstrated by real time reverse transcriptase polymerase chain reaction (RT-PCR). Of note, the renal mRNA abundance of TRPM7 remained unaltered. This observation of diverse regulation of TRPM6 in kidney and colon raises an intriguing and still unresolved question concerning the different regulatory mechanisms of magnesium reabsorption and absorption in these tissues [13]. Moreover, the elaborate regulation of TRPM6 transcription in response to body magnesium balance needs to be further elucidated.

Regulation by intracellular factors

As TRPM6 plays a crucial role in magnesium balance, the effect of intracellular magnesium on its activity has been studied using flash photolysis of the photolabile magnesium chelator DM-nitrophen, which directly alters cytosolic magnesium concentration in a spatially uniform manner [14]. By this approach, Voets et al. [14] demonstrated that intracellular magnesium can directly reduce TRPM6 activity. This regulation may function as a feedback mechanism to control magnesium reabsorption and absorption. Since a large part of intracellular magnesium is normally chelated by ATP, it is also possible that the channel is directly controlled by Mg\(^{2+}\).ATP. Thus, the role of magnesium, ATP and Mg\(^{2+}\).ATP in regulating TRPM6 channel activity needs to be more clearly defined.

Recently, receptor for activated C-kinase (RACK1) has been identified by yeast two-hybrid analysis as another intracellular regulatory factor for TRPM6, which specifically binds to the TRPM6 \(\alpha\)-kinase domain [38**]. RACK1 is a ubiquitously expressed protein that is involved in various cellular functions [39–41]. In kidney, however, RACK1 is predominantly expressed in DCT, where active magnesium reabsorption occurs, supporting its physiological role in TRPM6 regulation [38**]. It has been shown that direct protein–protein interaction is a common mechanism of ion channel regulation [34,42,43]. In this respect, RACK1 inhibits TRPM6 channel activity in an autophosphorylation-dependent manner. Moreover, RACK1 is involved in the magnesium-dependent suppression of TRPM6 channel activity, since the knockdown of endogenous RACK1 by small interfering RNA (siRNA) significantly reduced the magnesium-dependent inhibition of the TRPM6 current [38**]. This suggests a novel feedback regulatory model of TRPM6 channel activity through which the autophosphorylation of T1851 activates the inhibitory effect of RACK1 and subsequently blocks TRPM6-mediated magnesium influx to avoid magnesium overload during epithelial magnesium reabsorption (Fig. 1). Interestingly, the association between RACK1 and TRPM6 is a dynamic process regulated by protein kinase C (PKC), suggesting that PKC modulates the regulatory capacity of RACK1 on channel activity [38**].

Regulation by the fused \(\alpha\)-kinase domain

The most remarkable feature of TRPM6, and its closest homologue TRPM7, is the intracellular C-terminal domain containing an active threonine/serine kinase, which belongs to the atypical family of eukaryotic \(\alpha\)-kinases [18,44]. Currently, the central question, with respect to this distinct combination of a channel and a kinase domain within a single protein, is the role of this \(\alpha\)-kinase domain on channel activity. Although the \(\alpha\)-kinases share no sequence homology with conventional kinases, structural alignment of the recently crystallized \(\alpha\)-kinase domain in TRPM7 with the cAMP-dependent kinase (PKA) indicates that the majority of structural elements, sequence motifs, and the position of key functional amino acid residues important for catalysis are similar in both \(\alpha\)-kinases and PKA [45,46]. The (auto)phosphorylation activity of the fused \(\alpha\)-kinase domain has been established by two recent studies, which showed that TRPM6 is able to phosphorylate itself and TRPM7 [38**,47]. Furthermore, T1851 has been identified as a major site of autophosphorylation within the TRPM6 \(\alpha\)-kinase domain [38**]. Previously, it has been demonstrated that the balance between phosphorylation and dephosphorylation operates as a key regulator of ion channel activity [48,49]. Several phosphorylation mutants, however, including the TRPM6 delta kinase mutant, the phosphotransferase-deficient K1804R mutant and the nonautophosphorylated T1851A mutant display similar currents to wild-type TRPM6 [38**]. These findings suggest that the \(\alpha\)-kinase domain and its (auto)phosphorylation events are not essential for TRPM6 channel activation. The inhibitory action of RACK1, however, requires a functional \(\alpha\)-kinase domain, particularly the autophosphorylated state of T1851. Additionally, an increase in intracellular magnesium concentration gradually enhances the autophosphorylation of TRPM6, while the magnesium sensitivity of the autophosphorylation is significantly reduced in the T1851A mutant. Consistently, channel activity of the nonautophosphorylated T1851A mutant is less sensitive to intracellular magnesium than wild-type TRPM6 [38**]. These data suggest that the \(\alpha\)-kinase domain may function as the intracellular magnesium sensor regulating TRPM6 channel activity in an indirect manner via other proteins, such as RACK1. Collectively, these data show that the fused \(\alpha\)-kinase domain is indeed an elaborate functional element of TRPM6. Considering the known diverse functions of the \(\alpha\)-kinase domain of TRPM7 [15,18,50–53], however, the role of the TRPM6 \(\alpha\)-kinase domain in
Figure 1 Overview of the molecular regulation of TRPM6

An epithelial cell from the distal convoluted tubule (DCT) showing the molecular effects of dietary magnesium, estrogen, tacrolimus, cyclosporin A, intracellular magnesium, receptor for activated C-kinase (RACK1) and epidermal growth factor (EGF) on TRPM6 regulation. Dietary magnesium, estrogen, tacrolimus and cyclosporin A control TRPM6 gene transcription. Extracellular acidic pH decreases the magnesium permeability of TRPM6. Intracellular magnesium directly regulates TRPM6 channel activity. RACK1 exerts its effect in a T1851 autophosphorylation-dependent manner, thereby initiating a feedback regulation on TRPM6 channel activity to control TRPM6-mediated magnesium influx. EGF is synthesized in DCT cells and subsequently released to the blood compartment, where it stimulates TRPM6 channel activity via activation of the basolateral EGF receptor.

regulation of channel activity and subsequent physiological events certainly requires further investigation.

**Regulation by pH**

Chronic metabolic acidosis is known to result in renal magnesium wasting, whereas chronic metabolic alkalosis exerts the opposite effect [54]. Accordingly, Nijenhuis et al. [54] demonstrated that renal TRPM6 expression in kidney is decreased by chronic metabolic acidosis, but increased by chronic metabolic alkalosis. In addition to its effect on TRPM6 transcriptional regulation, hydrogen ions have been shown to directly influence TRPM6 channel activity. Extracellular pH directly affects the channel characteristics of TRPM6. Li et al. [16] demonstrated that lowering the extracellular pH significantly decreases divalent permeability by competing with divalent cations for binding sites. This leads to potentiation of the monovalent current through TRPM6. Further investigations identified E1024/E1029 in the TRPM6 pore region as the crucial site mediating the pH sensitivity of TRPM6 [16,20]. Interestingly, the corresponding residues in the pore region of TRPM7 are also key determinants of the pH sensitivity of this channel [16]. Together, these findings may provide the molecular basis for metabolic acidosis-induced renal magnesium wasting.

**Regulation by other factors**

Tacrolimus and cyclosporin A are currently the most potent immunosuppressive drugs used for therapeutic purposes such as organ transplantsations, and to treat allergies and autoimmune diseases. These drugs, however, can also induce significant side effects, including a disturbance of the magnesium balance as reflected by hypomagnesemia [55,56]. Nijenhuis et al. [55] showed that TRPM6 expression in kidney is downregulated by tacrolimus treatment, which may provide a molecular explanation for the tacrolimus-induced hypomagnesemia. Furthermore, in a rat kidney epithelial NRK-52E cell line, TRPM6 expression is decreased by cyclosporin A in a dose-dependent manner. It has been further pointed out that this process is mediated by inhibition of c-Fos transcription. This finding may explain the observed magnesiuria in patients treated with cyclosporin A leading to hypomagnesemia [56].

In addition to the drug-induced hypomagnesemia, magnesium deficiency has also been observed in patients with Gitelman’s syndrome, an autosomal recessive disorder caused by mutations in the thiazide-sensitive sodium chloride cotransporter (NCC) gene [57–59]. In line with this, hypomagnesemia develops in NCC-knockout mice, an animal model of Gitelman’s
syndrome, and during chronic hydrochlorothiazide administration, which blocks NCC activity. Recently, it has been reported that TRPM6 is downregulated upon NCC inhibition by hydrochlorothiazide and in NCC-knockout mice [57]. Thus, the downregulation of TRPM6 may represent a general mechanism involved in the pathogenesis of hypomagnesemia accompanying NCC inhibition or inactivation [57].

Together, these data support the crucial role of TRPM6 in active transepithelial magnesium reabsorption and highlight TRPM6 as a potential target for pharmacological treatment of various magnesium-deficient disorders.

Conclusion
Active magnesium reabsorption in the kidney plays a central role in magnesium homeostasis. As the gatekeeper of this process, tight control of TRPM6 activity enables the organism to adjust the magnesium balance in health and disease. Here, we provided an overview of the molecular regulation of TRPM6 by various means including magnesiotropic hormones, intracellular factors and the fused α-kinase domain (Table 1) [13,14,16,20*, 29**,38**54–57]. Detailed information concerning the trafficking and gating of TRPM6, however, remains basically elusive. Future investigations into these aspects will further increase our understanding of magnesium homeostasis and ultimately should pave the way to diagnoses and management of the corresponding diseases.

Acknowledgements
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Table 1 Regulation of TRPM6 activity

<table>
<thead>
<tr>
<th>Regulatory factor</th>
<th>Effect</th>
<th>Affected process</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dietary magnesium</td>
<td>↑</td>
<td>Transcription*</td>
<td>[13]</td>
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<tr>
<td>Estrogen</td>
<td>↑</td>
<td>Transcription (in kidney)</td>
<td>[13]</td>
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<tr>
<td>Tacrolimus</td>
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<td>Transcription (in kidney)</td>
<td>[55]</td>
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<td>Cyclosporin A</td>
<td>↓</td>
<td>Transcription (in NRK-52E cell)</td>
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<tr>
<td>Chronic metabolic acidosis</td>
<td>↑</td>
<td>Transcription (in kidney)</td>
<td>[54]</td>
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<tr>
<td>Chronic metabolic alkalosis</td>
<td>↑</td>
<td>Transcription (in kidney)</td>
<td>[54]</td>
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<tr>
<td>NCC inhibition</td>
<td>↓</td>
<td>Transcription (in kidney)</td>
<td>[57]</td>
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<tr>
<td>Intracellular magnesium and magnesium nucleotides</td>
<td>↓</td>
<td>Channel activity</td>
<td>[14]</td>
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<tr>
<td>EGF</td>
<td>↑</td>
<td>Channel activity</td>
<td>[29]</td>
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<tr>
<td>RACK1</td>
<td>↑</td>
<td>Channel activity</td>
<td>[38**]</td>
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<tr>
<td>Fused α-kinase domain</td>
<td>Indirect</td>
<td>Channel activity</td>
<td>[38**]</td>
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<tr>
<td>Acidic pH</td>
<td>↑</td>
<td>Monovalent current</td>
<td>[16.20*]</td>
</tr>
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* Magnesium restriction significantly increases renal TRPM6 mRNA levels, whereas a magnesium-enriched diet increases TRPM6 mRNA expression in colon.
† and ‡ mean increase or decrease, respectively, of the indicated processes including gene transcription, divalent permeability and channel activity of TRPM6. NRK-52E, a rat kidney epithelial cell line endogenously expressing TRPM6; NCC, thiazide-sensitive NaCl cotransporter; EGF, epidermal growth factor; RACK1, receptor for activated C-kinase.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
•• of outstanding interest
Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 426–427).
This study characterized the crucial amino acids in the pore region for magnesium and calcium permeability and pH sensitivity in TRPM6 and TRPM7.


32. Reynolds NA, Wagstaff AJ. Cetuximab: in the treatment of metastatic colo-


In this study, RACK1 has been identified as the first interacting protein of TRPM6. RACK1 binds to TRPM6 α-kinase domain and inhibits TRPM6 channel activity in an autophosphorylation-dependent manner. These data suggest a novel feedback regulatory mechanism of TRPM6 channel activity to control TRPM6-mediated magnesium influx.


41. Sklan EH, Posoly E, Soreq H. RACK1 has the nerve to act: structure meets function in the nervous system. Prog Neurobiol 2006; 78:117–134.


