Characterization of the Protein Kinase Activity of TRPM7/ChaK1, a Protein Kinase Fused to the Transient Receptor Potential Ion Channel*

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Lillia V. Ryazanova, Maxim V. Dorovkov, Athar Ansari, and Alexey G. Ryazanov‡

From the Department of Pharmacology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Channel-kinase TRPM7/ChaK1 is a member of a recently discovered family of protein kinases called α-kinases that display no sequence homology to conventional protein kinases. It is an unusual bifunctional protein that contains an α-kinase domain fused to an ion channel. The TRPM7/ChaK1 channel has been characterized using electrophysiological techniques, and recent evidence suggests that it may play a key role in the regulation of magnesium homeostasis. However, little is known about its protein kinase activity. To characterize the kinase activity of TRPM7/ChaK1, we expressed the kinase catalytic domain in bacteria. ChaK1-cat is able to undergo autophosphorylation and to phosphorylate myelin basic protein and histone III on serine and threonine residues. The kinase is specific for ATP and cannot use GTP as a substrate. ChaK1-cat is insensitive to staurosporine (up to 0.1 mM) but can be inhibited by rottlerin. Because the kinase domain is physically linked to an ion channel, we investigated the effect of ions on ChaK1-catalytic activity. The kinase requires Mg\(^{2+}\) (optimum at 4–10 mM) or Mn\(^{2+}\) (optimum at 3–5 mM), with activity in the presence of Mn\(^{2+}\) being 2 orders of magnitude higher than in the presence of Mg\(^{2+}\). Zn\(^{2+}\) and Co\(^{2+}\) inhibited ChaK1-cat kinase activity. Ca\(^{2+}\) at concentrations up to 1 mM did not affect kinase activity. Considering intracellular ion concentrations, our results suggest that, among divalent metal ions, only Mg\(^{2+}\) can directly modulate TRPM7/ChaK1 kinase activity in vivo.

A new family of protein kinases that do not display sequence homology to conventional eukaryotic protein kinases has been recently identified (1, 2). When mammalian and Caenorhabditis elegans elongation factor-2 kinases (eEF-2\(^{\alpha}\) kinases) were cloned, it was found that they do not display sequence homology to any conventional eukaryotic protein kinase (1). However, their catalytic domains appeared to be homologous to the catalytic domain of myosin heavy chain kinase A from Dicyostelium (3–5). Two more protein kinases with the same type of catalytic domain have been subsequently identified in Dictyostelium and have been called myosin heavy chain kinases B and C (6, 7). This new family of protein kinases was named α-kinase, because the existing evidence suggests that these protein kinases phosphorylate amino acids located within α-helices (2). This is different from conventional protein kinases that phosphorylate amino acids located within loops, turns, or regions with irregular structure (8). The α-kinase catalytic domain is characterized by several conserved motifs, which are different from the distinguishing sequence motifs that are found in conventional protein kinases (2). Surprisingly, the recently determined three-dimensional structure of the α-kinase catalytic domain revealed that, despite the lack of sequence homology, α-kinases have a fold that is very similar to conventional eukaryotic protein kinases (9).

Five more human proteins with the α-kinase domain have been identified and cloned (2, 10, 11). Unexpectedly, it was found that two of these proteins, which we initially named melanoma and kidney α-kinases and subsequently renamed channel-kinases 1 and 2 (ChaK1 and ChaK2), contained domains that are homologous to members of the transient receptor potential (TRP) family of ion channels (10, 11). Three other laboratories independently cloned ChaK1 and named it ChaK (9), TRP-PLIK (12), or LTRPC7 (13). The TRP family of ion channels consists of various cation channels with diverse cellular functions that can be subdivided into three subfamilies: TRP-classic (TRPC), TRP-vanilloid (TRPV), and TRP-melastatin (TRPM) (reviewed in Refs. 14–16). According to recently suggested unified nomenclature for TRP channels (16), ChaK1 and ChaK2 are now called TRPM7 and TRPM6 respectively.

The electrophysiological properties of the TRPM7/ChaK1 channel have been recently characterized (12, 13). It was found that the TRPM7/ChaK1 channel is permeable to Ca\(^{2+}\) and Mg\(^{2+}\), is inhibited by Mg\(^{2+}\) or Mg-ATP (13), and is inactivated by phosphatidylinositol 4,5-bisphosphate hydrolysis (17). A distinctive current that is believed to be mediated by TRPM7/ChaK1 and that can be inhibited by Mg\(^{2+}\), has been characterized in several types of cells (13, 18–20). A recent study suggests that TRPM7/ChaK1 may represent a novel ion channel mechanism for cellular trace metal ion entry into vertebrate cells (21). Another recent study suggests that TRPM7/ChaK1 may play a key role in the regulation of Mg\(^{2+}\) homeostasis (22).

Despite the extensive characterization of TRPM7/ChaK1 channel activity, little is known about TRPM7/ChaK1 kinase activity besides its ability to autophosphorylate and to phos-
phorylate myelin basic protein (10, 12, 22). In addition, the functional inter-relationship between the α-kinase domain and the ion channel domain in TRPM7/ChaK1 is still unclear.

This work provides the first detailed characterization of the protein kinase activity of channel-kinase TRPM7/ChaK1. We investigated the biochemical properties of the TRPM7/ChaK1 kinase catalytic domain and, in particular, the effect of various divalent metal ions on TRPM7/ChaK1 kinase activity. Our results suggest that, among divalent metal ions that TRPM7/ChaK1 can permeate, only Mg²⁺ can directly modulate TRPM7/ChaK1 kinase activity in vivo.

EXPERIMENTAL PROCEDURES

Materials—Buffer reagents and other chemicals were obtained from Sigma. Rottlerin and staurosporine were dissolved in Me₂SO. Radioisotopes were from PerkinElmer Life Sciences. PCR reagents and restriction enzymes were from Invitrogen and Roche Applied Science. Myelin basic protein (MBP) and calmodulin were a kind gift of Dr. Donald Wolff (Department of Pharmacology, UMDNJ). eEF-2 purification as well as expression and purification of eEF-2 kinase were performed as described previously (5).

Expression and Purification of ChaK1-cat—We expressed the C-terminal part of the TRPM7/ChaK1 containing kinase catalytic domain as a fusion with maltose binding protein (ChaK1-cat). Two fusion proteins were produced: ChaK1-cat (short), containing the last 462 amino acids (1403-1864) of TRPM7/ChaK1 (GenBank accession number AF346629), and ChaK1-cat (long), containing the last 767 amino acids. All experiments were carried out with both the short and long forms of ChaK1-cat, and essentially no difference was observed in any of the experiments. Experimental data presented in Figs. 1-7 were obtained with the short form of ChaK1-cat.

To produce ChaK1-cat (short form), a DNA fragment corresponding to the TRPM7/ChaK1 kinase domain was obtained by PCR from HeLa marathon-ready cDNA (Clontech) using the following primers: mkh-C3, 5'-TGTTAGTACACCATCTCATCGACCAAGTGGAAA-3' and mkh-CR, 5'-TTGTTAGTACACCATCTCATCGACCAAGTGGAAA-3'. The PCR product was inserted into a pCR II-TOPO vector (Invitrogen) by TA cloning and then subcloned into a pMAL-p2x vector (New England Biolabs) using EcoRI restriction sites to obtain the resulting construct, pMAL-mkhC 3.15. The expression and purification of ChaK1-cat (short form) was performed as follows. Escherichia coli DH5α cells harboring the pMal-mkhC 3.15 construct were grown at 37 °C in 1 liter of LB medium supplemented with 2g/liter glucose (with ampicillin, 100 μg/ml) to A₆₀₀ of 0.5 and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cells were then grown for an additional 6 h at 37 °C. All of the following procedures were carried out at 4 °C. The cells were pelleted, resuspended in 10 ml of buffer A (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 10 mM β-mercaptoethanol, and 20% (w/v) glycerol) containing complete protease inhibitor (Roche Applied Science), and dissolved in an 80-ml solution of 8M urea in buffer A. The sample was sonicated and centrifuged at 30,000 × g for 30 min. The supernatant was dialyzed overnight against buffer A. After dialysis, the sample was centrifuged again at 30,000 × g for 30 min. The supernatant was loaded onto an amylose (New England Biolabs) column (10 ml) equilibrated with buffer A containing complete protease inhibitor (Roche Applied Science). Elution was performed by a step gradient using buffer A plus 10 mM maltose.

To produce ChaK1-cat (long form), a DNA fragment corresponding to the whole C-terminal part of TRPM7/ChaK1 starting right after the sixth trans-membrane domain was obtained by PCR using TRPM7/ChaK1 cDNA in pCR II-TOPO as a template and the following primers: chak1-C5-blunt, 5'-AATGTGTATTTACAAGTGA-3' and chak1-CR, 5'-TTATAACATCGACAGAACAGATTAGTGATTCTGATTCT-3'. The PCR fragment was inserted into a pCR II-TOPO vector (Invitrogen) by TA cloning and then subcloned into a pMAL-p2x vector (New England Biolabs) using XmnI and XbaI restriction sites to obtain the resulting construct, pMAL-mkhC 3.15. The expression and purification of ChaK1-cat (short form) was performed as follows. Escherichia coli DH5α cells harboring the pMAL-mkhC 3.15 construct were grown at 37 °C in 1 liter of LB medium supplemented with 2g/liter glucose (with ampicillin, 100 μg/ml) to A₆₀₀ of 0.5 and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cells were then grown for an additional 6 h at 37 °C. All of the following procedures were carried out at 4 °C. The cells were pelleted, resuspended in 10 ml of buffer A (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, 10 mM β-mercaptoethanol, and 20% (w/v) glycerol) containing complete protease inhibitor (Roche Applied Science), and dissolved in an 80-ml solution of 8M urea in buffer A. The sample was sonicated and centrifuged at 30,000 × g for 30 min. The supernatant was dialedyzed overnight against buffer A. After dialysis, the sample was centrifuged again at 30,000 × g for 30 min. The supernatant was loaded onto an amylose (New England Biolabs) column (10 ml) equilibrated with buffer A containing complete protease inhibitor (Roche Applied Science). Elution was performed by a step gradient using buffer A plus 10 mM maltose.

**Fig. 1. Analysis of purified ChaK1-cat by electrophoresis and gel filtration.** A, SDS-PAGE analysis of purified ChaK1-cat. 1.5 μg of affinity-purified enzyme was subjected to electrophoresis on 7.5% SDS-polyacrylamide gels and stained with Coomassie Blue R-250. B, gel filtration chromatography of ChaK1-cat. 15 μg of affinity-purified recombinant protein was subjected to gel filtration on a Bio-Sil CEC 250-5 high pressure liquid chromatography column (Bio-Rad) equilibrated with buffer (25 mM Tris-HCl, pH 7.6, 1 mM MgCl₂, 0.5 mM dithiothreitol, and 150 mM NaCl). Fractions of 0.5 ml each were collected. ChaK1-cat activity was determined in each fraction using MBP as a substrate. Inset, the column was calibrated with standard molecular weight marker proteins (thyroglobulin, 670,000; IgG, 158,000; ovalbumin, 44,000; myoglobin, 17,000; and vitamin B₁₂, 1350).
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RESULTS

To investigate the protein kinase activity of TRPM7/ChaK1, we expressed its C-terminal part containing kinase catalytic domain as a fusion with maltose binding protein (MBP). SDS-PAGE analysis of affinity-purified fusion protein (ChaK1-cat) showed a major band migrating at about the 95-kDa position (Fig. 1A). To determine whether ChaK1-cat is a multimer in solution, gel filtration was performed. ChaK1-cat eluted as a single kinase activity peak corresponding to a protein with the molecular mass of ~300 kDa (Fig. 1B). This molecular mass is higher than the expected molecular mass of ChaK1-cat dimer (190 kDa); however, it is consistent with a dimer if the molecule is elongated. In fact, according to x-ray analysis, ChaK1 crystallizes as a dimer that has an unusually elongated structure (9). Therefore, active ChaK1-cat is likely to be a dimer in solution.

Affinity-purified ChaK1-cat was able to undergo autophosphorylation and also phosphorylated MBP and histone H3 (Fig. 2A). ChaK1-cat appears to be specific for ATP and cannot utilize GTP as a phosphate donor. Neither autophosphorylation nor phosphorylation of MBP was observed when ATP was substituted in the reaction with GTP (Fig. 2B). Fig. 2C represents the time course of phosphorylation of MBP by ChaK1-cat. Incorporation of $^{32}$P into MBP was linear during the first 15 min. To analyze the effect of autophosphorylation on kinase activity, ChaK1-cat was preincubated with unlabeled ATP for 20 min at 30 °C before the addition of MBP and $\gamma^{32}$P-ATP. As can be seen from Fig. 2D, preincubation of ChaK1-cat with ATP resulted in inhibition of subsequent autophosphorylation but did not affect the kinetics of MBP phosphorylation, suggesting that autophosphorylation does not affect ChaK1-cat kinase activity.

Phosphoamino acid analysis revealed that autophosphorylation of ChaK1-cat and phosphorylation of MBP occur predominantly on serine (Fig. 3, A and B). Phosphorylation of threonine in both cases was barely detectable (Fig. 3, A and B). In contrast to autophosphorylation and phosphorylation of MBP, phosphorylation of histone H3 occurred at threonine (60%) and serine (40%) (Fig. 3C). As can be seen in Fig. 3D, eEF-2 kinase, another α-kinase that is known to phosphorylate its substrate on threonine, indeed phosphorylated eEF-2 exclusively on threonine.

Considering the unique nature of TRPM7/ChaK1 as being protein kinase covalently linked to an ion channel, there is a possibility that TRPM7/ChaK1 kinase activity can be regulated by the ions that permeate the channel. Because TRPM7/ChaK1...
channel is permeable not only to Ca$^{2+}$ but also to other divalent metal ions, such as Mg$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, Ni$^{2+}$, and Co$^{2+}$ (21) and is Mg$^{2+}$-regulated (13), we investigated the effect of Mn$^{2+}$ and other divalent metal ions on ChaK1-catalysed kinase activity. As can be seen in Fig. 4, A and B, ChaK1-cat requires the presence of Mg$^{2+}$ for its activity, and the optimal concentration of Mg$^{2+}$ for both phosphorylation of MBP by ChaK1-cat and its autophosphorylation is ~4–10 mM.

We found that Mn$^{2+}$ dramatically activates ChaK1-cat. Analysis of the effect of Mn$^{2+}$ at concentrations between 0.1 and 10 mM revealed that the stimulatory effect of Mn$^{2+}$ is noticeable at 0.5 mM and is maximal at ~3.5 mM (Fig. 4C and D). Both autophosphorylation and phosphorylation of MBP are strongly activated by Mn$^{2+}$ (Fig. 4C). Phosphorylation of MBP by ChaK1-cat at 3.5 mM Mn$^{2+}$ is stimulated ~70-fold (Fig. 4D). Autophosphorylation in the presence of Mn$^{2+}$ also caused a noticeable shift in the electrophoretic mobility of ChaK1-cat. In the presence of Mn$^{2+}$, Mg$^{2+}$ is not required for the phosphorylation reaction, and at 3.5 mM Mn$^{2+}$ and 10 mM Mg$^{2+}$ kinase activity was slightly lower than in the presence of 3.5 mM Mn$^{2+}$ alone (Fig. 4A). Thus, ChaK1-cat requires either Mg$^{2+}$ or Mn$^{2+}$; however, the kinase activity is ~2 orders of magnitude higher in the presence of Mn$^{2+}$ than in the presence of Mg$^{2+}$. As can be seen in Fig. 4E, phosphorylation of histone H3 by ChaK1-cat was also strongly increased when MnCl$_2$ was added to the reaction mixture.

It is known that some protein kinases can change their specificity for phosphorylated amino acid when a reaction is performed in the presence of Mn$^{2+}$ instead of Mg$^{2+}$. For example, phosphorylase kinase acts as a serine-specific kinase in the presence of Mg$^{2+}$ but acts as a tyrosine kinase in the presence of Mn$^{2+}$ (22). Therefore, we performed phosphoamino acid analysis of autophosphorylated ChaK1-cat as well as MBP and histone H3 phosphorylated by ChaK1-cat in the presence of Mn$^{2+}$. The addition of Mn$^{2+}$ does not affect specificity of ChaK1-cat for phosphorylated amino acid. When autophosphorylation of ChaK1-cat and phosphorylation of MBP were performed in the presence of Mn$^{2+}$, phosphorylation of both serine and threonine increased with >90% of $^{32}$P incorporated into serine (data not shown). Similarly, in the presence of Mn$^{2+}$, phosphorylation of both threonine and serine in histone H3 was significantly increased. The activity and specificity of eEF-2 kinase were not affected by the addition of Mn$^{2+}$ (data not shown).

Other divalent cations that we tested cannot substitute for Mn$^{2+}$ in activating ChaK1-cat. In fact, Zn$^{2+}$ and Co$^{2+}$ strongly inhibit phosphorylation of MBP by ChaK1-cat (Fig. 5). Both Zn$^{2+}$ and Co$^{2+}$ at concentrations of 500 μM completely inhibited MBP phosphorylation by ChaK1-cat (Fig. 5).

The addition of Ca$^{2+}$ (0.001, 0.01, 0.1, and 1 mM) or EGTA (2 mM) did not have any effect on the kinase activity of ChaK1-cat (Fig. 6A). However, in the presence of calmodulin, the addition of 0.1 or 1 mM Ca$^{2+}$ led to significant inhibition of MBP phosphorylation by ChaK1-cat (Fig. 6A). Similarly, 1 mM Ca$^{2+}$ in the presence of calmodulin inhibited the phosphorylation of histone H3 (Fig. 6B). However, the addition of Ca$^{2+}$ and calmodulin did not have any effect on the autophosphorylation of ChaK1-cat (Fig. 6, A and B).
To further analyze the inhibitory effect of Ca\(^{2+}\)/calmodulin on the phosphorylation of MBP by ChaK1-cat, the reaction was performed at various concentrations of MBP and ChaK1-cat. It was found that the inhibitory effect of Ca\(^{2+}\)/calmodulin did not depend on the concentration of ChaK1-cat (Fig. 6C). However, the magnitude of the inhibitory effect of Ca\(^{2+}\)/calmodulin was clearly dependent on the concentration of MBP, and at high concentrations of MBP, this inhibitory effect was not observed (Fig. 6D). These results suggest that the inhibitory effect of Ca\(^{2+}\)/calmodulin on the phosphorylation of MBP by ChaK1-cat is either because of calmodulin interaction with MBP or because of competition between calmodulin and MBP for binding to the kinase.

We also investigated the effect of monovalent cations on ChaK1-cat kinase activity. Both K\(^+\) and Na\(^+\) produced significant inhibitory effects on phosphorylation of MBP by ChaK1-cat at concentrations above 100 mM (Fig. 7A), suggesting that high ionic strength inhibits ChaK1-cat.

We analyzed the sensitivity of ChaK1-cat to some known inhibitors of conventional protein kinases. Interestingly, ChaK1-cat appears to be resistant to staurosporine, which did not produce any inhibitory effect even at the concentration of 100 \(\mu\)M (Fig. 7B). Another protein kinase inhibitor, rottlerin, inhibits ChaK1-cat with an IC\(_{50}\) of \(-35\) \(\mu\)M (Fig. 7, B and C).

**DISCUSSION**

In this work, we provide the first detailed characterization of the protein kinase activity of the channel-kinase TRPM7/ChaK1. Among \(\alpha\)-kinases, the kinase activity of only eEF-2 kinase and *Dictyostelium* myosin heavy chain kinases A, B, and C has been characterized previously. Interestingly, in contrast to eEF-2 kinase and myosin heavy chain kinases, which phosphorylate their substrates exclusively on threonines (24), we have found that phosphorylation of MBP by ChaK1-cat, as well as its autophosphorylation, occur predominantly on serine (Fig. 3). ChaK1-cat can also efficiently phosphorylate histone H3 on serine and threonine residues (Fig. 3). Thus, TRPM7/ChaK1 can be considered a serine/threonine-specific protein kinase.

According to gel filtration, ChaK1-cat exists in solution as a dimer. This is consistent with the crystallographic analysis of ChaK1. It was found that the kinase domain of ChaK1 forms a dimer in the crystal as a consequence of the exchange between monomers of a 27-residue helical segment (9). The oligomeric structure of ChaK1 in *vivo* is unclear. It is believed that TRP channels exist *in vivo* as tetramers (14–16). Therefore, if kinase and channel domains of TRPM7/ChaK1 are, indeed, functionally and physically linked *in vivo*, the kinase might exist as a “dimer of dimers.”

We found that ChaK1-cat is specific for ATP and cannot use GTP as a substrate for the phosphorylation reaction (Fig. 2B). Although there are conventional protein kinases that are specific for ATP, many of them can utilize GTP as well as ATP (25). Analysis of ChaK1 structure (9, 26) suggests an explanation of why this kinase is specific for ATP. It appears that one of the GTP oxygens would repel Glu-1718 located in the nucleotide-binding site, making binding of GTP unlikely. Interestingly, this glutamate (Glu-1718) is absolutely conserved among \(\alpha\)-ki-
nases (26), and therefore other α-kinases are likely to be specific for ATP. In fact, we found that eEF-2 kinase cannot use GTP as a substrate.2

We found that staurosporine, a compound that interferes with ATP binding and inhibits most conventional protein kinases, does not have any effect on the kinase activity of ChaK1-cat at concentrations up to 0.1 mM (Fig. 7B). This result was surprising given the structural similarity between ChaK1 and conventional protein kinases (9). However, detailed structural analysis (26) suggests an explanation for this result. In conventional protein kinases, there is substantial rearrangement of the residues in the active site to accommodate the bulky staurosporine molecule. However, in ChaK1, there is a salt bridge between Glu-1718 and Lys-1646 in the back of the hydrophobic pocket, which limits the flexibility of the binding site and makes staurosporine binding unlikely. Because amino acids making this salt bridge are conserved in all α-kinases (26), it is likely that other α-kinases will also not be inhibited by staurosporine. In fact, it was shown previously that eEF-2 kinase is relatively resistant to staurosporine (27).

Rottlerin, another compound known to inhibit protein kinases (see e.g. Ref. 28), inhibits both autophosphorylation and phosphorylation of MBP by ChaK1-cat with an IC50 of ~35 μM (Fig. 7, B and C). Rottlerin similarly inhibits eEF-2 kinase (27) and therefore may be a general inhibitor of α-kinases.

Considering the unique nature of TRPM7/ChaK1 as a protein kinase covalently linked to an ion channel, there is a possibility that TRPM7/ChaK1 kinase activity can be regulated by the ions that permeate the channel. Therefore, we investigated the effect of various metal ions on the ChaK1-cat kinase activity. TRPM7/ChaK1 is permeable to both Ca2+ and Mg2+ (13) as well as other divalent ions such as Mn2+, Zn2+, Ni2+, and Co2+ (21).

The possible effect of Mg2+ on TRPM7/ChaK1 kinase activity is particularly interesting not only because Mg2+ can permeate this channel but also because TRPM7/ChaK1 channel activity is known to be modulated by intracellular Mg2+ (13), and, according to a recent report, TRPM7/ChaK1 may play a key role in the regulation of magnesium homeostasis in vertebrates (22). In addition, it was found recently that mutations in a closely related channel-kinase, TRPM6/ChaK2, lead to hypomagnesemia, a disease characterized by a low Mg2+ concentration in blood serum (29, 30), suggesting that this channel-kinase also plays an important role in the regulation of Mg2+ homeostasis.

We found that ChaK1-cat kinase has Mg2+ optimum between 4 and 10 mM (Fig. 4, A and B). This magnesium optimum is very similar to other α-kinases as well as many conventional protein kinases. Conventional protein kinases have two Mg2+ binding sites, one with high affinity (dissociation constant <0.1 mM) and another with lower affinity (dissociation constant >1 mM) (31, 32). Occupation of both sites with Mg2+ is required for optimal activity of the majority of conventional protein kinases, and therefore they usually have a Mg2+ optimum between 2 and 10 mM. Cyclic AMP-dependent protein kinase (PKA) is an exception to this rule in that binding of the second Mg2+ is inhibitory (reviewed in Ref. 31), and, therefore, PKA has a Mg2+ optimum below 1 mM. Because the Mg2+ concentration in the cytoplasm is usually around 1 mM, ChaK1 kinase potentially can be regulated in vivo by changes in Mg2+ concentration, particularly if TRPM7/ChaK1 functions as a Mg2+ channel, and therefore can affect the local concentration of Mg2+ in the vicinity of the kinase.
Because TRPM7/ChaK1 may also function as a Ca\(^{2+}\)/H\(_{11001}\) channel (12, 13), we investigated the effect of Ca\(^{2+}\)/H\(_{11001}\) on ChaK1-cat kinase activity. Our results demonstrate that the TRPM7/ChaK1 kinase domain was not sensitive to changes in calcium concentration in the range of 0.001–1 mM (Fig. 6A), and, therefore, it is unlikely that this kinase can be directly regulated by calcium flowing through the channel. However, at high concentrations of Ca\(^{2+}\), calmodulin inhibited ChaK1-mediated phosphorylation of MBP and histone H3 (Fig. 6, A and B). This effect could either be due to the binding of calmodulin to the substrate or because of competitive inhibition of the enzyme by calmodulin, as it was not observed when substrate concentration was increased (Fig. 6D). A similar effect was described by Wolff et al. (33), who found that binding of Ca\(^{2+}\)/calmodulin to histones 1, 2A, and 2B inhibited histone dephosphorylation by bovine brain phosphatase.

TRPM7 is permeable to other divalent metal ions, and it was recently suggested that it can provide an ion channel mechanism for cellular entry of trace metal ions such as Mn\(^{2+}\), Zn\(^{2+}\), and Co\(^{2+}\) (21). We investigated the effect of these metal ions on the kinase activity of ChaK1-cat and discovered that Mn\(^{2+}\) dramatically stimulates phosphorylation of MBP and histone H3 by ChaK1 (Fig. 4). Zn\(^{2+}\) and Co\(^{2+}\), in contrast to Mn\(^{2+}\), inhibited ChaK1-cat activity (Fig. 5). Interestingly, the effect of divalent metals on ChaK1-cat kinase activity is very similar to the effect of these metal ions on several conventional protein kinases, particularly tyrosine kinases (34–40). It was reported for various tyrosine kinases, such as insulin receptor tyrosine kinase (35) and EGF receptor tyrosine kinase (34, 36), that their activity can be dramatically stimulated in the presence of Mn\(^{2+}\). There are also serine/threonine kinases that can be activated by Mn\(^{2+}\) (37, 40). This effect is most likely because of the binding of Mn\(^{2+}\) instead of Mg\(^{2+}\) to the second metal-binding site (31, 39). At the same time, it was demonstrated that binding of Zn\(^{2+}\) or Co\(^{2+}\) at this second binding site is inhibitory in tyrosine kinases (39). A striking similarity between the effects of divalent metal ions on ChaK1-cat activity and tyrosine kinases suggests that the catalytic mechanism of phosphotransfer in α-kinases and conventional protein kinases may be similar.

Is it possible that the effects of divalent metal ions that we observed are involved in the regulation of TRPM7/ChaK1 ki-
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nase activity in vivo. Because Mn$^{2+}$, Zn$^{2+}$, and Co$^{2+}$ produce their effects on ChaK1-cat activity at a very high concentration that is not observed at physiological conditions (Figs. 4 and 5), and Ca$^{2+}$ has no effect on ChaK1-cat activity (Fig. 6), these ions are unlikely to be involved in the modulation of TRPM7/ChaK1 kinase activity in vivo. Therefore, according to our results, among divalent metal ions, only Mg$^{2+}$ can potentially regulate TRPM7/ChaK1 kinase activity in the cell, which is also consistent with a recent report by Schmitz et al. (22). If, indeed, TRPM7/ChaK1 functions in vivo as a Mg$^{2+}$ channel, then it is possible that the kinase activity of TRPM7/ChaK1 is regulated by Mg$^{2+}$ flux through its ion channel.

According to the recent report, the TRPM7/ChaK1 channel plays an important role in the regulation of vertebrate cellular Mg$^{2+}$ homeostasis (22). It was suggested that the TRPM7/ChaK1 channel acts as a primarily Mg$^{2+}$-permeant channel and represents cellular Mg$^{2+}$ uptake mechanism (22). However, the function of TRPM7/ChaK1 kinase is unclear. The kinase domain is not required for TRPM7/ChaK1 channel gating, although it may play some role in modulation of channel activity (22). The kinase domain can also play a role in mediating the interaction of TRPM7/ChaK1 with other cellular proteins such as phospholipase C$\beta$ (12, 17). Clearly, the identification of TRPM7/ChaK1 kinase substrates is essential to understanding the physiological role of this kinase. The unique properties of TRPM7/ChaK1 protein kinase described in this paper, such as its dramatic activation by manganese and its insensitivity to staurosporine, will be important tools that may help to distinguish the activity of TRPM7/ChaK1 from other kinases and to identify its natural substrates.

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