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Drosophila MagT1 is upregulated by PKC activation

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ABSTRACT

Magnesium transporter subtype 1 (MagT1) is a newly discovered and evolutionarily conservative magnesium membrane transporter with channel like properties. Previous reports have demonstrated that MagT1 is important to cellular magnesium homeostasis. In this study, we investigated whether drosophila MagT1 (dMagT1) was functionally regulated by PKC activation *in vitro*. With patch clamping, we have observed that whole cell currents of wild type dMagT1 were magnesium selective and non-voltage dependent when expressed in a human neuroblastoma SH-SY5Y cell line. Furthermore, dMagT1 currents were significantly increased in cells treated with a non specific PKC activator PMA, but not in cells treated with the inactive form of PMA, 4α-PMA. Lastly, we have demonstrated that upregulation of dMagT1 currents by PKC activation involves specific PKC phorsphorylation sites in dMagT1. Of all three dMagT1 mutants created for testing the putative PKC phorsphorylation sites, dMagT1-S35A displayed a significant increase of whole cell currents while dMagT1-S100A and -S108A were not affected by PKC activation. Thus, we have demonstrated that dMagT1 is a magnesium selective transporter with basic biophysical characters similar to its mammalian homolog and can be functionally upregulated by PKC activation. Both dMagT1 Ser100 and Ser106 are equally important to this PKC-dependent modulation, therefore the most likely molecular sites for PKC phorsphorylation. The data presented here may establish a general regulatory mechanism for MagT1 by PKC activation.

1. Introduction

Magnesium transporter subtype 1 (MagT1) is a newly discovered and evolutionarily conservative magnesium transporter. Primary biophysical properties of MagT1 include highly selective to magnesium over other physiological divalent cations, non-voltage dependent gating and magnesium saturable [1]. The expression pattern of MagT1 is widely distributed in tissues, thus may be critical to cellular magnesium homeostasis [1].

Indeed, MagT1 mutations have been shown to cause a rare form of human T cell immune deficiency, mutants of MagT1 lost the ability to mediate rapid elevation of intracellular free magnesium in T cells during specific immune activation [2]. Moreover, experimental knock-out of *magt1* caused early stop of embryonic development in zebra fish, which could be rescued by over expression of other membrane magnesium transporters [3]. However, how MagT1 is regulated remains largely hypothetic.

It has been suggested that MagT1 possesses putative phorsphorylation sites on its N-terminal region for PKC by preliminary sequence analysis [1,3]. What more important is that these sites are highly conservative, signaling significant regulatory pathways may exit for MagT1. This has prompted us to investigate the molecular mechanism of MagT1 regulation by PKC activation. We have used drosophila MagT1 (dMagT1) to address the question. The primary results described here have, for the first time, presented solid evidence that dMagT1 has basic biophysical properties similar to its mammalian homolog and its function is indeed upregulated by PKC activation *in vitro*.

2. Materials and methods

2.1. Preparation of dMagT1 plasmid and site-directed mutagenesis

The wild type dMagT1 plasmid (GenBank: NM_135360.3) was obtained from Berkeley Drosophila Genome Project (BDGP, USA) and reconstruct into a mammalian expression vector pcDNA3.1. Through MagT1 sequence alignment among drosophila (NP_609204.2), zebra fish (NP_955994.1), mouse (NP_001177338.1) and human (NP_115497.4), we have identified Ser35, Ser100 and Ser108 as putative PKC phorsphorylation sites located in dMagT1 N-terminal region (Table 1). Site-directed mutagenesis was utilized to create point mutations in dMagT1 corresponding to these sites with

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Table 1
Sequence alignment of the N-terminal region of MagT1 homologues. There are up to three putative sites (in italics) for PKC phorsphorylation in the N-terminal region of dMagT1. The serine and threonine in bond black are putative PKA phorsphorylation sites [1].

Drosophila	31	GLSL*S*EKVQN	LVDMNAKKPL	LRFNGPKFRE	YVKSAPRNY**S**
Danio rerio	27	ETLL*S*EKVSQ	MMEWV*S*KRAV	VRLNGEKFKR	LVRAHPRNY**S**
Mus musculus	67	EMVL*S*EKVSQ	LMEWANKRPV	IRMNGDKFRR	LVKAPPRNY**S**
Homo sapiens	34	EMVL*S*EKVSQ	LMEWTNKRPV	IRMNGDKFRR	LVKAPPRNY**S**
Drosophila	71	MIVML**T**ALAP	SRQCQICRHA	HDEFAIVA*N*S	YRFSSTYSNK
Danio rerio	67	VIVMF**T**ALQP	QRQCGVCRQA	DEEYQILA*N*S	WRYSSAF*T*NR
Mus musculus	107	VVVMF**T**ALQL	HRQCVVCKQA	DEEFQILA*N*S	WRYSNAF*T*NR
Homo sapiens	74	VIVMF**T**ALQL	HRQCVVCKQA	DEEFQILA*N*S	WRYSSAF*T*NR

Quick Change® kit (Merck). Three mutations of dMagT1-S35A, -S100A and -S108A were constructed where the serine was replaced with an alanine, respectively. Plasmids of wild type and mutant dMagT1 were validated via sub-cloning and sequencing assay (Invitrogen, Shanghai).

2.2. Selection of a cell line for dMagT1 expression

To minimize the interference of endogenous MagT1 in the cell line used for dMagT1 expression, we conducted a screening to evaluate endogenous MagT1 expression levels in cell lines commonly used for ion channel study with real time PCR. The expression levels of MagT1 in CHO, HEK293 and HeLa cells were analyzed and compared to mouse ventricular cardiac muscles where MagT1 is expressed [1]. All three cell lines have substantial expression levels of endogenous MagT1, 120% in HEK293, 51% in CHO and 44% in HeLa when compared to the control ($n = 3$, data not shown), thus are not appropriate for this study. On the other hand, we found a human neuroblastoma SH-SY5Y cell line with a low MagT1 expression level, only 5% to that of the control ($n = 3$, data not shown). More importantly, SH-SY5Y cell line is known to express PKCs, thus is suitable for this study [4]. The culture media for SH-SY5Y cells contains (in 500 ml): DMEM 4.95 g, L-Glutarnine 0.3 g, 10% $NaHCO_3$ 10 ml, FBS 50 ml, distilled water 440 ml.

2.3. Patch-clamp recording

SH-SY5Y cells were seeded onto small cover slips in culture dishes and kept in a CO_2 incubator for overnight before transient transfection. The plasmid of dMagT1 (0.5 μg) and GFP (0.5 μg) were mixed in a total volume of 2 μl for transient transfection with a FuGene HD kit (Roche). For the induction of PKC activation, 10 μM of Phorbol-12-myristate-13-acetate (PMA) or its inactive form 4α-PMA (Sigma–Aldrich) was added into the culture media and incubated for 1 h just before the patch clamping experiments. The recording window was selected to be 20–28 h after transfection. Cells with green fluorescent were selected for patch clamping under room temperatures.

The recording system is consisted of a HEKA EPC-10 amplifier with a head stage, a micromanipulator (MPC-325, Sutter), an upright microscope (BX51WI, Olympus) with fluorescent capability and a plastic recording camber with a gravity-aided perfusion system (home made). Borosilicon glass pipettes (VitalSense Scientific Instruments, China) were prepared with a programmable horizontal puller (P-97, Sutter). The tip of pipette was heat polished with a microforge (MF-900, Narishige). The final resistance of an open pipette was ~10 MΩ with normal saline. The pipette solution contains (in mM): KCl 140, EGTA 10, $MgCl_2$ 1, HEPES 10, K_2ATP 5 (pH 7.3). Bath solution contains (in mM): NaCl 100, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, Glucose 10, Sucrose 87 (pH 7.3). In bath solutions contained higher magnesium or calcium, sucrose was replaced in equal osmole. All chemicals, reagents, culture ware and supplies used in this study were purchased from Sigma–Aldrich or elsewhere as stated.

Voltage protocols were generated by the Patchmaster software (HEKA) and delivered to the recording pipette connected to the head stage of the amplifier. Signals were sampled in 2 kHz and filtered by 1 kHz online. Giga seals were formed between pipette tip and cell membrane. Capacitance compensation was adjusted to ~80%. All data were stored in a personal computer for off line analysis with Clampfit (Axon, Molecular Device) and Origin (Microcal). Unpaired student *t*-test was employed for statistic analysis.

3. Results and discussion

3.1. Characterization of dMagT1 currents

MagT1 is a magnesium transport protein widely expressed in different tissues and evolutionarily conservative [1,3,5]. Drosophila MagT1 (NP_609204.2) displays a sequence similarity of 72% to the human MagT1 (NP_115497.4). Although phylogenetically distant from mammal, drosophila has long been established as an excellent model to address biology inquiries at all levels. To test if dMagT1 shares the basic biophysical features with its mammalian homolog, we measured the whole-cell currents of SH-SY5Y cells transfected with dMagT1. The whole cell currents were induced by a family of pulse steps from −125 to 25 mV at an increment of 15 mV while the holding was at −15 mV. The whole cell currents evoked by voltage-steps were instant upon voltage changes (Fig. 1A). The linear *I–V* relationship constructed from whole-cell recordings clearly indicates voltage independent nature of the currents (Fig. 1B). The amplitudes of whole-cell current were increased by and corresponsive to the elevation of bath magnesium from 1 to 20 mM (Fig. 1B). A partial saturation of the whole currents was reached when bath magnesium was increased to 30 mM (Fig. 1B). Meanwhile, the reversal potential (E_r) for the whole cell currents was shifted positively and an averaged change in E_r was 21 mV when bath magnesium was increased from 1 to 30 mM (Fig. 1C). A theoretic shifting of E_r for an ideal magnesium conductance is calculated by Nernst equation to be 32 mV when extracellular magnesium changes from 1 to 30 mM, thus the whole cell currents observed here were not perfectly but largely magnesium selective.

On the other hand, whole-cell currents were not significantly changed when bath calcium was changed from 2 to 30 mM (Fig. 1D). Also, magnesium-dependent changes of whole-cell currents were not observed in untransfected cells (data not shown). Given that SH-SY5Y cells have very low expression levels of endogenous MagT1 and the over expression nature of the vector used for dMagT1, we conclude that the whole-cell currents observed in SH-SY5Y cells merely represent the dMagT1 activity that retains basic biophysical characteristics of its mammalian homolog [1].

3.2. Effect of PKC activation on dMagT1 currents

There are up to four tentative PKC modulation sites in the N-terminal region of human MagT1 (hMagT1) as suggested in the first

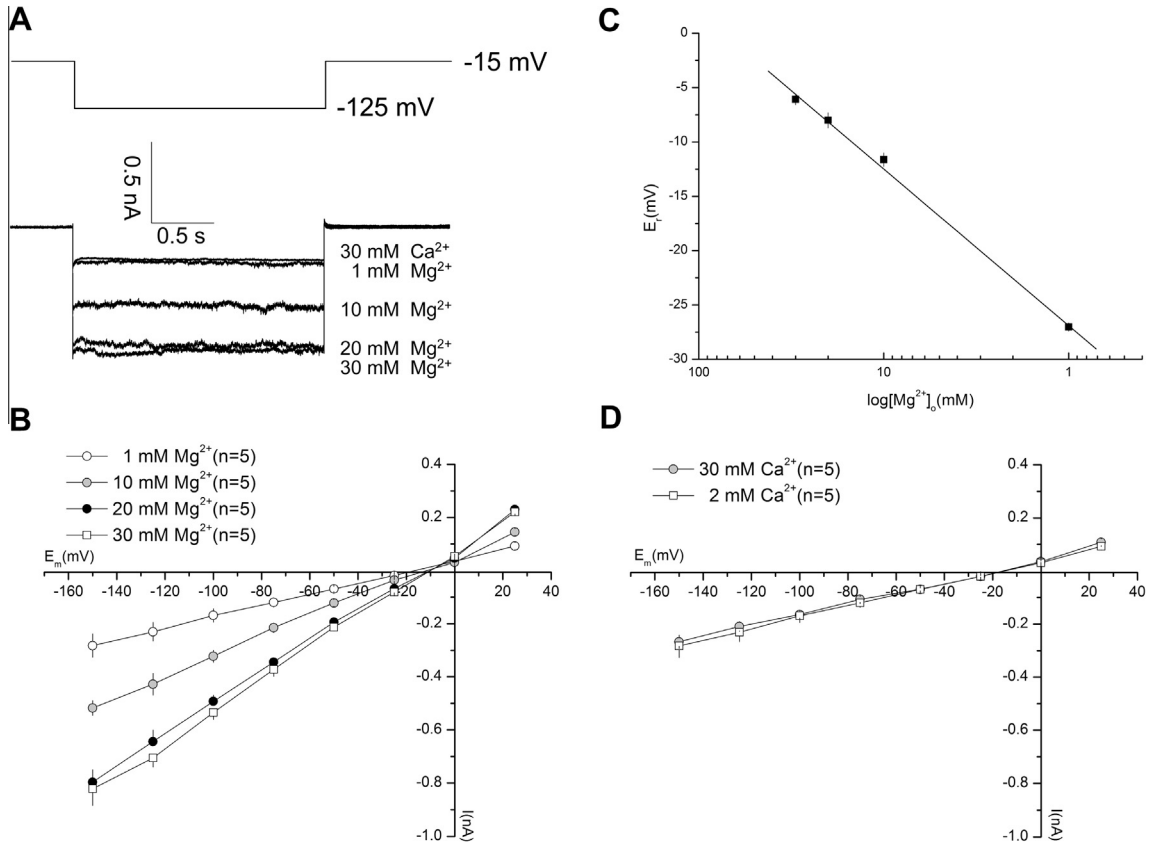

Fig. 1. Characteristics of dMagT1 whole cell currents. Wild type dMagT1 was transient transited to SH-SY5Y cells. Representative inward currents were displayed in responding to magnesium bath concentrations when a voltage step from −15 to −125 mV was applied (A). *I–V* curves derived from whole cell recordings show voltage-independent, magnesium dependent and saturable characteristics (B). Relationship between reversal potential E_r of whole cell currents from cells transfected with dMagT1 and bath magnesium concentration (C). *I–V* curves show no significant changes of dMagT1 whole cell currents when bath calcium was changed from 2 to 20 mM (D).

study of MagT1 [1]. Yet, effect of PKC activation on Magt1 function has not been seen in literatures so far. Through MagT1 sequence alignment among drosophila, zebra fish, mouse and human, we have found that these putative PKC phorsphorylation sites are evolutionarily conservative (Table 1). This has provided us with the merit to investigate if PKC activation plays a role in regulating MagT1 function.

After pre-incubation with a non-specific PKC activator PMA, whole cell currents in SH-SY5Y cells transfected with dMagT1 were significantly increased (Fig. 2A, B), while whole cell currents in untransfected cells showed no significant changes form the control (data not shown). The whole cell currents were not significantly changed in cells transfected with dMagT1 after 1 h pre-incubation of 4α-PMA, an inactive form of PMA (Fig. 2C). Thus, we have observed a functional enhancement of dMagT1 mediated by PKC activation *in vitro*. Given the conservative nature of PKC signaling paradigm from yeast to mammalian, our finding may signify a general regulatory mechanism of MagT1 by PKC activation [6].

Up till now, there are more than 10 isoforms of PKC being revealed, which are classified into the conventional (cPKCs, 3), novel (nPKCs, 7) and atypical (aPKCs, 5) groups. All PKCs share a highly conservative catalytic kinase domain, but differ in regulatory motifs that confer specificity of regulatory input and/or subcellular localization to each isoform of the three groups [6]. Recent progress in functional modulation of ion channels by PKC has suggested that outcome of PKC regulation is isoform specific [7]. Under physiological conditions, the cPKCs and nPKCs can be activated by the increase of intracellular free calcium or DAG levels. PMA is a non-physiological membrane permeable PKC activator, capable of binding to the DAG-binding C1 regulatory domain in both cPKCs and nPKCs [6]. Despite the fact that SH-SY5Y cell line is known to express both cPKCs and nPKCs, we are not able to pin point which isoform of PKCs is involved in our observation due to the non-specific nature of PMA [4]. Nonetheless, our results have highlighted a new direction in understanding cellular magnesium homeostasis regulated by PKC activation.

The potential significance of functional enhancement of MagT1 by PKC activation may not be limited to cellular magnesium homeostasis because PKC also acts on cellular calcium homeostasis in tissues such as cardiac muscles. For example, activation of εPKC produced inhibition on cardiac α_{1C} L-type calcium channel via phorsphorylation of the Thr27 and Thr31 in the N-terminal region [8], while activation of α, βI, βII, υ, δ and θPKCs produced activation of the same channel via phorsphorylation of the C-terminal Ser1674 [9]. Moreover, the cardiac α_{1D} L-type calcium channel is the target of βII and εPKC-mediated phorsphorylation on the Ser81 in the C-terminal region, causing inhibitory effect [10]. Given the fact that both L-type calcium channels and MagT1 are co-expressed in various tissues and magnesium and calcium are natural antagonists for each other, it would be interesting to see if there is any crosstalk between cellular magnesium and calcium homeostasis governed by the activity of PKC. Since the molecular identity and subcellular localization of both MagT1 and L-type calcium channels are clearly defined, such investigation may be worthwhile to explore [11].

3.3. Identification of specific sites in dMagT1 for PKC modulation

Although it was suggested that human MagT1 has up to four consensus sites in the N-terminal region for PKC phorsphorylation

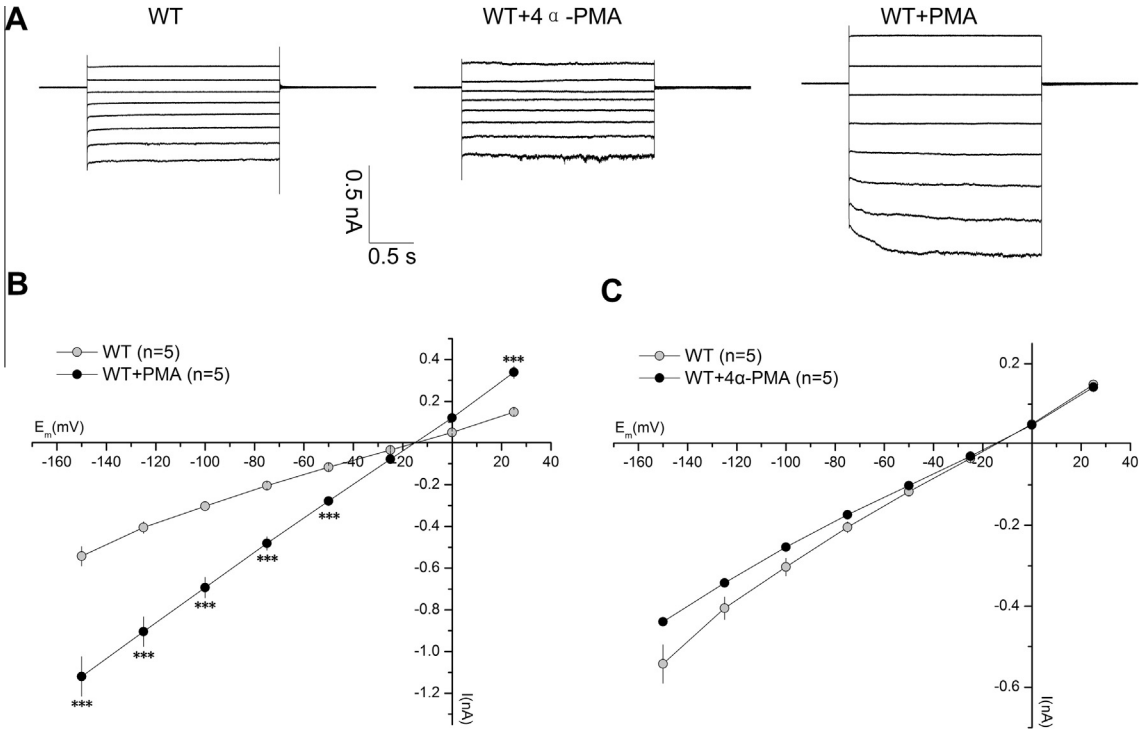

Fig. 2. Effect of PKC activation on dMagT1 currents. Representative dMagT1 whole cell currents were recorded from cells treated with 10 μM PMA or 10 μM 4α-PMA, respectively (A). Significant increases of dMagT1 whole cell currents were only seen in the PMA treated cells (B), but not in the 4α-PMA treated cells ($n = 5$, $^{**}p < 0.001$, $^{***}p < 0.0001$) (C).

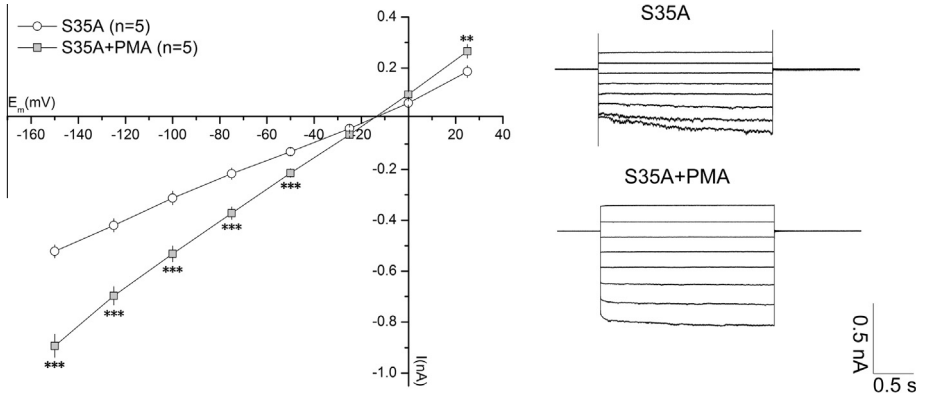

Fig. 3. Effect of PKC activation on dMagT1-S35A currents. *I–V* curves are derived from whole cell recordings in cells transfected with dMagT1-S35A. PMA treatment enhanced dMagT1 current significantly ($n = 5$, $^{***}p < 0.0001$).

[1], we have found that only three of them are evolutionarily conservative evidenced by the result of sequence alignment (Table 1). Thus, we determined to test whether each of the three sites was involved in the PKC modulation on dMagT1. Whole cell currents were recorded from cells transfected with dMagT1 mutants in the presence of 10 mM magnesium in bath. Whole cell currents of dMagT1-S35A were significantly increased in response to PMA stimulation, similar to that of wild type dMagT1 to PMA stimulation (Fig. 3A, B). On the other hand, neither dMagT1-S100A nor dMagT1-S108A responded to the PMA treatment; whole cell currents recorded from SH-SY5Y cells transfected with these two mutants were not significantly different from that of the control (Fig. 4A, B). Based on what we found here, we conclude that both dMagT1 Ser100 and Ser108, but Ser35, are directly related to the effect of PKC activation on dMgT1 currents under our experimental conditions and are most likely the sites for PKC phorsphorylation. Since PMA can activate cPKCs and nPKCs via binding to the DAG-site that is not possessed by aPKCs, our results cannot exclude the possibility that dMagT1 Ser35 is a suitable site for aPKCs.

Taken together, we have demonstrated that dMagT1 is a functional magnesium transporter with basic biophysical properties similar to its mammalian homolog. And function of dMagT1 can be upregulated by PMA-stimulated PKC activation *in vitro*. The Ser100 and Ser108 in the N-terminal region of dMagT1 are directly involved in the upregualtion of dMagT1 currents by PKC activation and equally important to this process. The data presented here may establish a general regulatory mechanism for MagT1 by PKC activation.

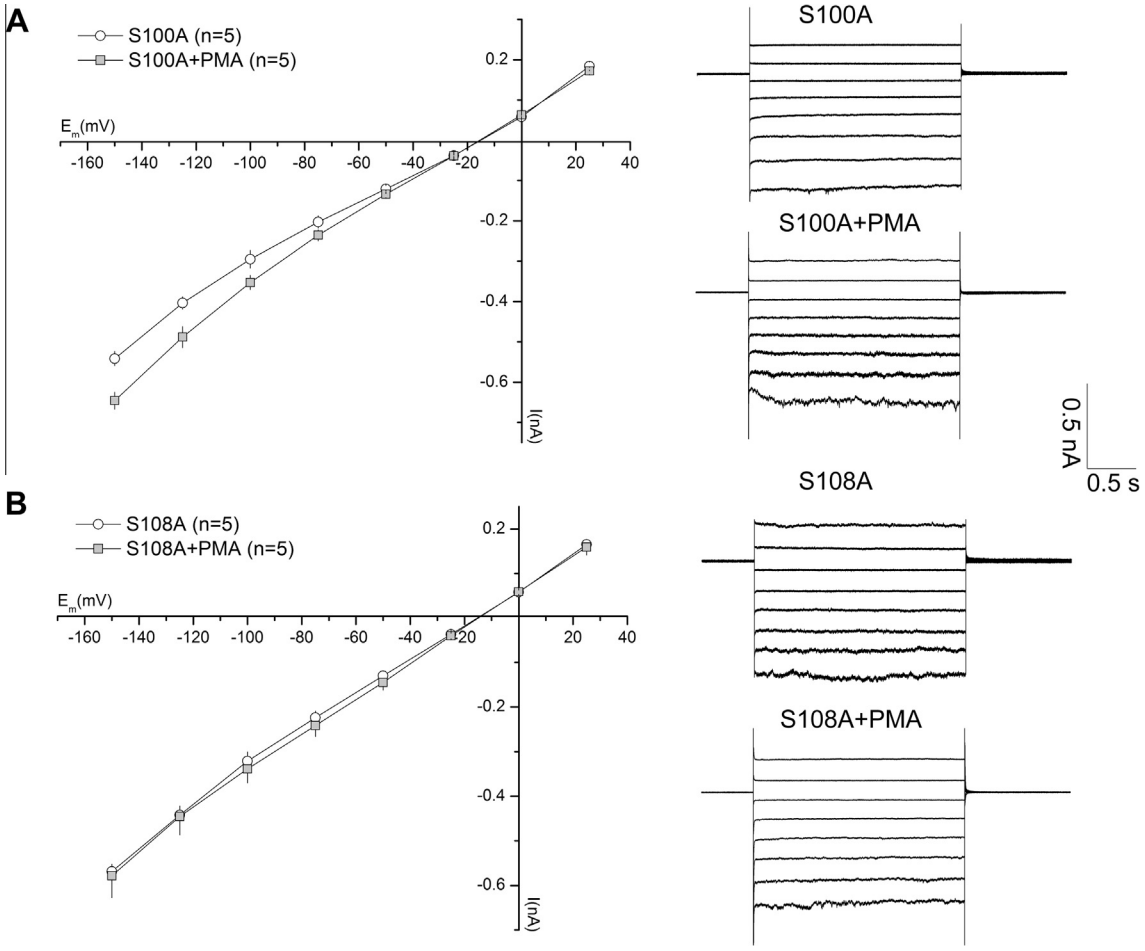

Fig. 4. Effect of PKC activation on dMagT1-S100A and -S108A currents. *I–V* curves are derived from whole cell recordings in cells transfected with dMagT1-S100A or -S108A. PMA treatment enhanced neither dMagT1-S100A nor -S108A currents significantly ($n = 5$).

Acknowledgments

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