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# Dual regulation of hEAG1 channels by phosphatidylinositol 4,5-bisphosphate

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ABSTRACT

The *ether-à-go-go1* (EAG1, Kv10.1) $K^+$ channel is a member of the voltage-gated $K^+$ channel family mainly expressed in the central nervous system and cancer cells. Membrane lipids regulate several voltage-gated $K^+$ channels but their influence on EAG1 channels has been poorly explored. Here we have studied the regulation of hEAG1 channels by phosphatidylinositol 4,5-bisfofate ($PIP_2$) by using different strategies to manipulate the levels of this lipid, and the patch clamp technique. We found that depletion of endogenous $PIP_2$ by activation of the voltage-sensing phosphatase from *Danio rerio* (Dr-VSP) or the human muscarinic type-1 receptor (hM1R) inhibits hEAG1 currents; however, the application of exogenous $PIP_2$ to increase the level of this lipid on the plasma membrane, also induced an inhibition of hEAG1. In summary, our results indicate that $PIP_2$ have dual effects on hEAG1 channels and its action as activator or inhibitor depends on its initial level on the plasma membrane.



## 1. Introduction

Human *ether-à-go-go1* (hEAG1, Kv10.1) $K^+$ channels are voltage-gated potassium channels mainly present in the central nervous system [1] and aberrantly expressed in numerous cancer cells types [2,3]. On depolarization, hEAG1 activate rapidly, reach a steady-sate level and do not inactivate [4]. hEAG1 channels are composed of four subunits, each consisting of six transmembrane segments (S1-S6) which form the voltage sensor domain (S1-S4) and the pore domain (S5-S6) [5]. The N- and C-terminus of hEAG1 contains the Per-Arnt-Sim (PAS) and cyclic nucleotide binding homology (cNBHD) domains respectively, which are important regulators of the voltage-dependent gating of the channel [6]. Like other voltage-gated $K^+$ channels, hEAG1 channels are regulated by diverse endogenous factors [7] including $Ca^{2+}$/calmodulin [8], hormones [9,10], divalent cations [11], and lipids [12,13].

Phosphatidylinositol 4,5-bisphosphate ($PIP_2$) is a minor phospholipid found in the inner leaflet of the plasma membrane that plays an important role as an intermediate molecule in multiple receptor signaling pathways [14]. Additionally, $PIP_2$ itself acts as a signaling molecule through direct interactions with different proteins [15]. Numerous ion channels have been reported to be $PIP_2$ sensitive [16,17]; however, some controversies exist due to the strategies used to manipulate the levels of this lipid [18,19]. In this study, we explored the $PIP_2$ regulation of hEAG1 channels by using different strategies to manipulate the $PIP_2$ levels in the plasma membrane.

## 2. Materials and methods

### 2.1. Reagents

Carbachol and poly-L-lysine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Both compounds were dissolved in water to make stock solutions of 25 mM (carbachol) and 3 mg/ml (poly-L-lysine). The stock solutions were diluted in bath solution to the final concentrations required for patch-clamp recordings. L-α-phosphatidylinositol 4,5-bisphosphate ($PIP_2$, natural from porcine brain) was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and prepared as previously described [20].

### 2.2. Cell culture and DNA expression

Human embryonic kidney 293 [HEK-293] cells (ATCC® CRL-1573™) were grown in 60-mm tissue culture dishes (Corning,

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Corning, NY, USA) at 37 °C in a humidified air atmosphere containing 5% $CO_2$. Cells were maintained in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (Corning Life Sciences, Manassas, VA, USA) and 1% (v/v) antibiotic-antimycotic solution (Sigma-Aldrich). HEK-293 cells were transiently transfected with cDNAs encoding human *ether-à-go-go1* channels (hEAG1, kindly provided by Dr. Michael C. Sanguinetti, University of Utah, USA), human muscarinic type-1 receptor (hM1R, kindly provided by Dr. José A. Sánchez-Chapula, Universidad de Colima, México), and the voltage-sensing phosphatase from *Danio rerio* (Dr-VSP, kindly provided by Dr. Yasushi Okamura, Osaka University, Japan) with the use of Lipofectamine (2000) reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. For electrophysiological recordings, cells were used 24 h after transfection. As a marker for successfully transfected cells, cDNA encoding the enhanced green fluorescent protein (EGFP) was co-transfected with the cDNAs of interest.

### 2.3. Electrophysiological recordings

Macroscopic current recordings in HEK-293 cells were performed at room temperature (22–24 °C) by using the whole-cell and inside-out configurations of the patch-clamp technique. Micropipettes were pulled from borosilicate glass capillary tubes (World Precision Instruments, Sarasota, FL, USA) on a programmable puller (Sutter Instruments, Novato, CA, USA) and had resistances of 1.5–2.5 MΩ when filled with the internal solution. Data acquisition and generation of voltage-clamp pulse protocols were carried out using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and a Digidata 1440A interface (Molecular Devices) controlled by the pCLAMP 10 software (Molecular Devices). Currents were filtered with a four-pole Bessel filter at 1 kHz and digitized at 10 kHz. The specific voltage-clamp protocols used are described in the corresponding Results sections or Figure legends. For whole-cell recordings, the external solution contained (in mM): 140 NaCl, 4 KCl, 1 $MgCl_2$, 10 HEPES, 1.8 $CaCl_2$, and 10 glucose (pH was adjusted to 7.4 with NaOH). The pipette solution contained (in mM): 130 KCl, 5 $MgCl_2$, 10 EGTA, 5 $K_2ATP$, and 10 HEPES (pH adjusted to 7.2 with KOH). Inside-out recordings were performed using symmetrical potassium concentrations (in mM): 120 KCl, 5 $K_2EDTA$, 7 $KH_2PO_4$, and 8 $K_2HPO_4$ (pH was adjusted to 7.4 with KOH). Solutions were applied using a Fast-Step Perfusion System (VC-77SP Warner Instruments, Hamden, CT, USA).

### 2.4. Data analysis and statistics

Data analysis was performed by using the pClamp 10 (Molecular Devices) and Origin 8.6 (OriginLab Corp., Northampton, MA, USA) software. Data are presented as the mean ± SEM. Statistical comparisons were made by unpaired or paired Student's t-test where applicable. Statistical significance was set at $p < 0.05$.

## 3. Results

### 3.1. $PIP_2$ dephosphorylation by Dr-VSP inhibited hEAG1 channels

To investigate the effects of $PIP_2$ on heterologously expressed hEAG1 channels we co-transfected the voltage-sensitive phosphatase from *Danio rerio* (Dr-VSP). Dr-VSP dephosphorylates $PI(4,5)P_2$ on the 5 position to give PI(4)P during depolarization. HEK-293 transfected only with hEAG1 cDNA exhibited the slowly rising and persistent outward currents characteristic of this channel (Fig. 1A). hEAG1 activates around −40 mV and increased almost linearly until 100 mV in the I-V relationship (Fig. 1C). When co-expressed with Dr-VSP, hEAG1 currents were inhibited when the voltage exceeded around +30 mV (Fig. 1B), consistent with the voltage-dependent activation of Dr-VSP [21].

To accurately measure the effect of $PIP_2$ dephosphorylation on hEAG1 channels, we applied a 3-step protocol: a 1-s pulse to −30 mV to exclusively activate hEAG1 (P1), followed by a 2-s pulse to +100 mV that activate both hEAG1 and Dr-VSP (P2), and finally a 1-s pulse to −30 mV (P3). Fig. 2A–B shows representative currents traces recorded with this 3-step protocol on cells expressing hEAG1 alone (Fig. 2A) or co-expressing hEAG1 and Dr-VSP (Fig. 2B). In cells co-expressing hEAG1 and Dr-VSP, the initial pulse to −30 mV (P1) elicited a slowly activating and persistent current that is followed by a fast increase in amplitude at +100 mV (P2); however, the current rapidly decreased by the concomitant activation of Dr-VSP, therefore, the amplitude at −30 mV in P3 is significantly smaller than in P1 (Fig. 2B). The ratio (P3/P1) of hEAG1 current amplitudes at −30 mV determined just before and right after the depolarization to +100 mV (P2), shows the remaining hEAG1 current after $PIP_2$ dephosphorylation; in hEAG1 expressing cells the P1/P3 ratio was of 0.97 ± 0.02, whereas it was of 0.1 ± 0.01 in cell co-expressing hEAG1 and Dr-VSP (Fig. 2C). A larger duration at −30 mV in P3 shows a substantial recovery of hEAG1 currents, presumably reflecting partial recovery of $PIP_2$ levels (Fig. 2D).

### 3.2. $PIP_2$ depletion by hM1R activation inhibited hEAG1 channels

We turned to another tool to decrease the $PIP_2$ levels in the plasma membrane and corroborated our previous results. We expressed hEAG1 channels together with the human M1 muscarinic receptor (hM1R). hM1R is a Gq-coupled receptor that activates phospholipase C (PLC) leading to $PIP_2$ depletion [22]. In our experiments, we used 10 μM carbachol to activate the hM1R. Fig. 3A–B shows representative recordings of hEAG1 currents sequentially obtained under control condition (Fig. 3A), and then after hM1R activation with carbachol (Fig. 3B). Carbachol application significantly reduced the maximal current amplitude at all the test potentials (Fig. 3C). Interestingly, the temporal course of hEAG1 currents after hM1R activation showed two patterns; some cells exhibited an initial slight increase in current amplitude before the inhibition (Fig. S1A), whereas others were inhibited just after carbachol application (Fig. S1B). These results confirm the inhibition of hEAG1 channels by decreasing the levels of $PIP_2$ in the plasma membrane.

### 3.3. Application of exogenous $PIP_2$ to excised inside-out membrane patches inhibited hEAG1 channels

Application of exogenous $PIP_2$ to excised inside-out membrane patches has been used to test for $PIP_2$ dependence of ion channels [23]. Recently, exogenously applied $PIP_2$ was reported to inhibits hEAG1 channels [13]. Here, we applied 5 μM $PIP_2$ to excised inside-out membrane patches expressing hEAG1 as an additional method to test for $PIP_2$ sensitivity of this channel under this condition. As previously reported, increasing $PIP_2$ levels by its exogenous application induced an inhibition of hEAG1 channels by 64.6 ± 3.4% (Fig. 4A–C). This result seems contradictory to that obtained by decreasing $PIP_2$ by two different methods in whole-cell (Figs. 1–3). Therefore, we evaluated the effects of decreasing $PIP_2$ levels on excised inside-out membrane patches. For this purpose, we tested the effect of 25 μg/ml of poly-L-lysine (a $PIP_2$ scavenger) on hEAG1 channels. Surprisingly, application of poly-L-lysine to excised inside-out membrane patches induced an inhibition of hEAG1 channels by 34.4 ± 3% (Fig. 4D–E), indicating that both increasing or decreasing $PIP_2$ levels in the plasma membrane inhibits hEAG1. The sequential application of poly-L-lysine and $PIP_2$ showed the fine

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