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PKA-mediated phosphorylation is a novel mechanism for levetiracetam, an antiepileptic drug, activating ROMK1 channels

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ABSTRACT

Levetiracetam (LEV) is an effective antiepileptic drug (AED) with distinct mechanism from the conventional AEDs. The major physiological function of ROMK1 channels is to maintain the resting membrane potential (RMP). In this study, we investigated the mechanisms underling LEV on ROMK1 channels. *Xenopus* oocytes were injected with mRNA to express the wild-type or mutant ROMK1 channels. Giant inside-out patch clamp recordings were performed to study the effect of LEV on these channels. LEV increased the activity of ROMK1 channels in a concentration-dependent manner and enhanced both wild-type and pH_i gating residue mutant (K80M) channels over a range of pH_i values. LEV activated the mutated channels at PIP_2-binding sites (R188Q, R217A and K218A) and PKC-phosphorylation sites channels (S4A, S183A, T191A, T193A, S201A and T234A). However, this drug failed to enhance the channel activity in the presence of PKA inhibitors and did not activate the mutants of PKA-phosphorylation sites on C-terminal (S219A, S313A) and the constructed mutants (S219D and S313D) that mimic the negative charge carried by a phosphate group bound to a serine. Our results demonstrated PKA-mediated phosphorylation is a novel mechanism for LEV activating ROMK1 channels. These findings show that LEV activates ROMK1 channels independently from pH_i and not via a PIP_2- or PKC-dependent pathway. The effects of LEV may come from the PKA-induced conformational change but not charge–charge interaction in ROMK1 channels. Enhancing the activity of ROMK1 channels may be an important molecular mechanism for the antiepileptic effects of LEV in restoring neuronal RMP to prevent seizure spreading.

1. Introduction

Levetiracetam (LEV; Keppra; (S)-α-ethyl-2-oxo-pyrrolidine acetamide) is a new antiepileptic drug (AED) [1,2]. Although the mechanism of this drug action remains to be fully elucidated, it appears to be different from the conventional AEDs. LEV dose not be modulated by γ-aminobutyratergic (GABAergic) facilitation, inhibition of either voltage-dependent Na^+ channels, or T-type voltage activate-gated Ca^{2+} currents [3]. It has been shown to inhibit the high-voltage-activated Ca^{2+} currents in the pyramidal neurons of rat hippocampal slices [4] and selectively involve in the subtype of N-type-Ca^{2+} channel [5]. It also decreases the sensitivity of $GABA_A$ receptor to Zn^{2+} and β-carbolines [3]. Moreover, LEV

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prolonged the duration of single action potentials, decreased the voltage-gated K^+ currents, and reduced the amplitude of action potentials elicited by repetitive stimulation in acutely isolated hippocampal neurons [6]. These findings signify the functional importance of the K^+ channels in the anti-seizure actions of LEV.

K^+ channels are found in virtually all cells. The basic function of K^+ channel is to regulate the resting membrane potential (RMP). One of the most functional important K^+ channels is inwardly rectifying K^+ (Kir) channels, which exist in various excitable and non-excitable cells. They maintained the RMP near the K^+ equilibrium potential [7]. Several Kir

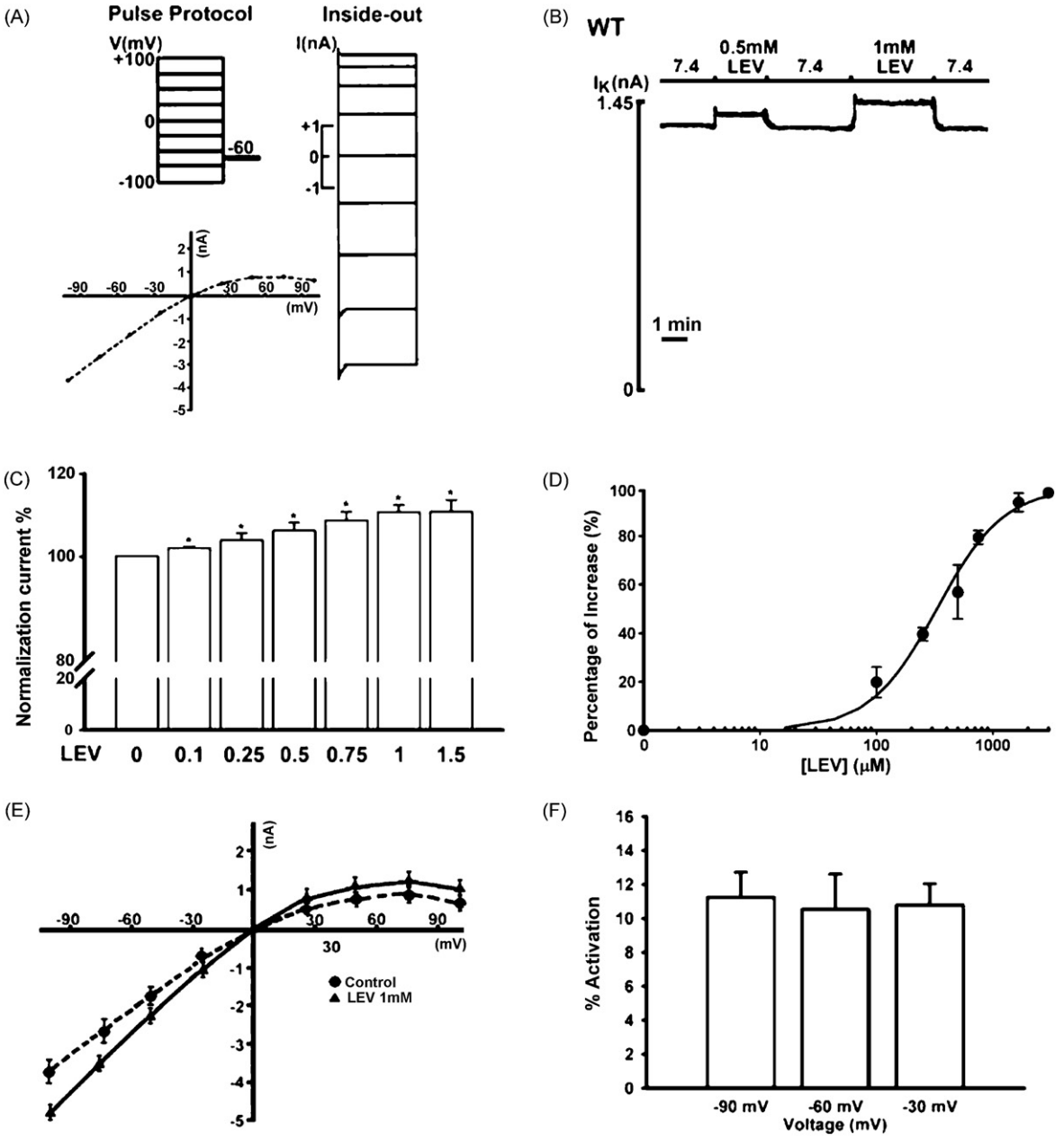

Fig. 1 – Levetiracetam (LEV) activates ROMK1 channel activity. All experiments are in FVPP at pH_i 7.4. (A) The pulse protocol and *I–V* curves for ROMK1 channels are presented. ROMK1 channels were expressed in *Xenopus* oocytes and the K^+ currents (I_k) recorded in inside-out giant patch clamped recordings. Voltage pulses were applied from −100 to +100 mV at 25 mV increments. The holding potential was −60 mV. ROMK1 channels showed a weak inwardly rectifying pattern. (B) An application of LEV (0.5 and 1 mM) significantly activates the ROMK1 current. (C) LEV (0.1–1.5 mM) activate ROMK1 channels currents in a concentration-dependent manner (n = 15 for each group). * indicates $P < 0.05$ by ANOVA. (D) LEV enhances ROMK1 channel activity with an EC_{50} = 363 μM. The activation of ROMK1 channels in the presence of LEV at a concentration of 1.5 mM was considered to be 100% and that at different concentration (0.1–1.5 mM) of this compound was compared. (E) The *I–V* relationship is shown in an inside-out patch before (circle) and after (triangle) application of LEV (1 mM). (F) The percentage of the activation affects of 1 mM LEV for ROMK1 channels over the voltage ranges from −90 to −30 mV. There was no significant interaction between the LEV effect and the membrane potential effect ($P > 0.05$).

channels have been shown to play a role in epileptic seizures [8–14]. The *weaver* mouse, which has a point mutation in the pore region of the Kir3 gene, induces seizure generation [9]. Kir6 channels have been report to involve in the control of generalized seizure activity during hypoxia [10]. Impaired extracellular potassium uptake by astrocytes through Kir2 channels is contributed to neuronal hyperexcitability and epileptogenesis in a mouse model of tuberous sclerosis complex [11]. The dysfunctional of Kir4 channels in glial cells increases the tendency of an epileptogenic activity in area CA1 of the rat hippocampus [12]. Reduced Kir current density and inward-rectification have been reported in brain slices from surgical specimens of patients with temporal lobe epilepsy [13,14]. ROMK1 (Kir1.1) channels have been expressed in the brain areas such as hippocampus and cortex and designated it to Kir1.1f [15,16]. The electrophysiological properties and physiological roles for ROMK1 channels in the brain (Kir1.1f) are similar to the isoforms in the kidney (Kir1.1a-c) [15]. Activation of ROMK1 channels reduces the action potential firing by a more hyperpolarized RMP, a higher value of spike threshold and increased the whole-cell conductance during cellular excitation in the hippocampal neurons which are important in the control of seizures generation [17]. The ROMK1 channels have been reported to be regulated by membrane phospholipid phosphatidylinositol bisphosphate (PIP_2), protein kinase A (PKA), protein kinase C (PKC) and intracellular pH (pH_i) [18–22]. In this study, we investigate that the effects of LEV in regulating ROMK1 channel activity by

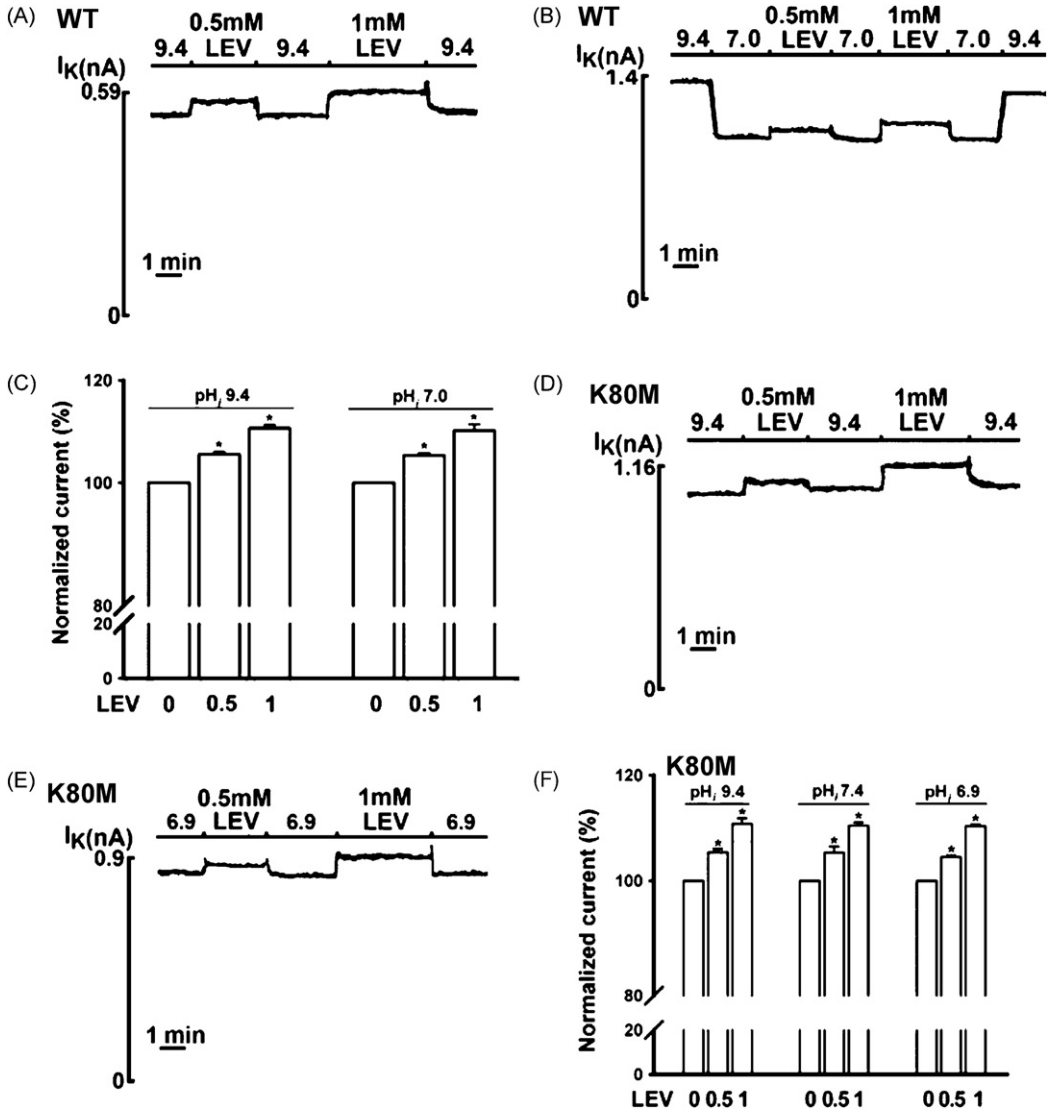

Fig. 2 – LEV activates ROMK1 activity is not depending on pH_i. (A) and (B) represent current trace from giant inside-out patches showing LEV (0.5 and 1 mM) enhancing ROMK1 channel activity at pH_i 9.4 and 7.0. (C) The percentage of the activation effects of LEV (0.5 and 1 mM) for ROMK1 channels at different pH_i (9.4 and 7.0, $n = 10$ for each group). * indicates $P < 0.05$ by ANOVA. As comparing the effect of LEV at pH_i 7.0 and 9.4, there is no significant difference for its activation of ROMK1 channels in different pH_i values ($P > 0.05$). (D) and (E) LEV (0.5 and 1 mM) activates K80M at pH_i 9.4 and 6.9. Experimental paradigm the same as in A. (F) The percentage of the activation affects of LEV (0.5 and 1 mM) for K80M channels at pH_i 9.4, 7.4 and 6.9 ($n = 5$ for each group). * indicates $P < 0.05$ by ANOVA.

using giant patch clamp recording in *Xenopus* oocytes. Our results demonstrated that LEV potentiates ROMK1 channel activity by a novel PKA-dependent mechanism.

2. Methods

2.1. Molecular biology

Site-directed mutagenesis was performed using a commercial mutagenesis kit (Stratagene Co., La Jolla, CA, USA) and confirmed by nucleotide sequencing as described [19]. mCAP RNAs of the wild-type and mutant channels were *in vitro* transcribed by using T7 RNA polymerase (Ambion Co., Austin, TX, USA) [19,21].

2.2. Oocytes preparation and injection

The experiment was approved by the Animal Ethics Committee of the National Taiwan University. Female *Xenopus laevis* frogs briefly were anesthetized by immersion in 0.1% 3-aminobenzoic acid ethyl ester. A few lobes of ovaries were removed after a small abdominal incision. The surgical incision was closed, and the frogs were allowed to recover from the anesthesia. *Xenopus* oocytes were treated with 2 mg/ml collagenase (Type I, Sigma, St. Louis, MO, USA) in the OR_2 solution containing (in mM) 82 NaCl, 2 KCl, 1 $MgCl_2$, and 5 Hepes (pH 7.4) for 90 min at room temperature (at 23–25 °C) to remove the follicular layer. After 10 washes of the oocytes with the OR_2 solution, the 30 ng of mRNA were injected into the oocytes (Dumont stage V–VI). The oocytes were then incubated at 18 °C in the ND96 solution containing (in mM) 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 Hepes, and 100 mg/l penicillin–streptomycin with 10 mg/ml geneticin added (pH 7.6). Channel activity was assessed 3–7 days post-injection.

2.3. Giant inside-out patch clamp recordings

Xenopus oocytes were injected with mRNA for the wild-type or mutant ROMK1 channels and giant inside-out patch clamp recordings were performed as described [19–21]. The pipette (extracellular) solution contains (in mM) 100 KCl, 2 $CaCl_2$, and 5 Hepes (pH 7.4). Bath (cytoplasmic) solution contains either 100 KCl, 5 Hepes (pH 7.4), 5 EGTA, and 1 $MgCl_2$ (Mg^{2+} solution) or 100 KCl, 5 Hepes, 5 EDTA, 4 NaF, 3 Na_3VO_4, and 10 $Na_4P_2O_7$ (FVPP solution) as indicated for each experiment. Inward K^+ currents at −60 mV holding potential were recorded in a chart recorder (23–25 °C) by using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Chart recording strips were scanned and analyzed in a computer for presentations.

2.4. Drug treatment and administration

Levetiracetam was kindly provided by UCB S.A. (Braine-l'Alleud, Belgium) and H89 (Sigma, St. Louis, MO, USA) were dissolved in distilled water. KT5720 (Sigma, St. Louis, MO, USA) and GF109203X (Sigma, St. Louis, MO, USA) were dissolved in dimethylsulfoxide. In the experiments of the role of PKC or PKA in regulating the effect of LEV on ROMK1 channels, the injected oocytes were pre-incubated for 5 min with GF109203X (10 μM) [23], H89 (10 μM) [21], and KT5720 (2 μM) [24] in ND96 solution before inside-out patch clamp recordings.

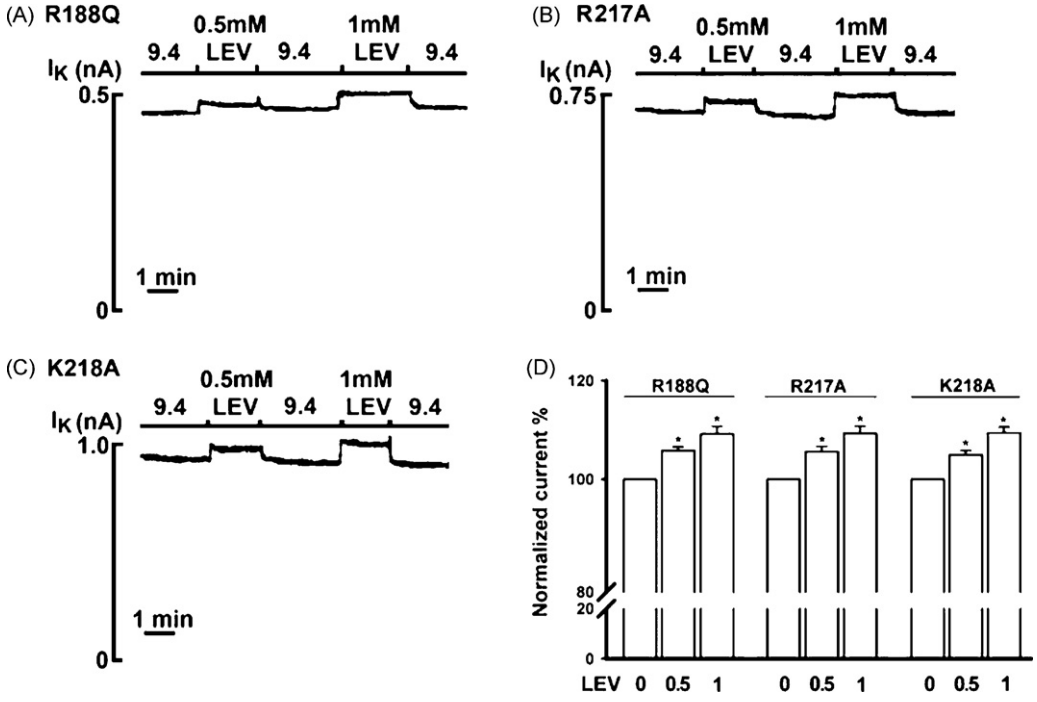

Fig. 3 – LEV activates ROMK1 channels may not involve in PIP_2-dependent pathway. (A)–(C) LEV (0.5 and 1 mM) activates the three PIP_2-binding sites mutants (R188Q, R217A, and K218A). (D) The percentage of the activation effect of LEV (0.5 and 1 mM) on the wild-type (WT) and the mutant channels of ROMK1 channels (R188Q, R217A and K218A, $n = 6$ for each group). * indicates $P < 0.05$ by ANOVA.

2.5. Data analysis

To examine the concentration-dependency of the effect of LEV on ROMK1 channel activity, the results were fitted to the Hill function, i.e., the absolute current amplitude = $(I_{max} \times [C]^n)/(EC_{50}^n + [C]^n)$, where [C] represents the LEV concentration, EC_{50} the concentration of LEV needed to increase channel activity by 50%, n the Hill coefficient, and I_{max} the maximal LEV-induced stimulation of ROMK1 channel currents. The values obtained are expressed as the mean ± S.E.M. with n being the number of oocytes tested. Statistical analysis was performed using analysis of variance (ANOVA) followed by Tukey–Kramer *post hoc* test when significance was reached in the ANOVA. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Activation of ROMK1 channels by LEV

Current–voltage (*I–V*) relationships (from −100 to +100 mV) and inward K^+ currents (holding at −60 mV) through ROMK1 channels were measured by using giant patch clamp recordings in *Xenopus* oocytes, first in "on-cell" configuration and subsequently in excised inside-out configuration into an FVPP bath solution that contained a mixture of the phosphatase inhibitors, fluoride, vanadate, and pyrophosphate (Fig. 1A). The solution prevents run-down of ROMK1 current probably by inhibiting both Mg^{2+}-dependent protein phosphatase as well as lipid phosphatase thus slowing channels dephosphorylation and membrane PIP_2 depletion [19,21]. The *I–V* relationship shows a characteristic weak inward-rectification for ROMK1 channels (Fig. 1A). Administration of LEV (0.5 and 1 mM) was observed to significantly activate the ROMK1 currents by (% of control) 5.88 ± 1.34 and 10.89 ± 1.22 ($n = 15$, $P < 0.05$, Fig. 1B). LEV increased channel activity in a concentration-dependent manner (Fig. 1C). The effect of LEV at a concentration of 1.5 mM was taken as the 100% value. This concentration-dependent effect of LEV was well fitted to a Hill function. The EC_{50} was calculated to be 363 μM (Fig. 1D). The steady state *I–V* relationships show an increase in the conductance of ROMK1 channels after application of LEV (1 mM) (Fig. 1E). The potentiation effect of LEV for ROMK1 channels was measured at membrane potentials between −90 and −30 mV. For ROMK1 channels, the percentage potentiation of LEV (1 mM) showed no significant difference across the voltages (−90 to −30 mV, $P > 0.05$, Fig. 1F).

3.2. Effect of pH_i on the enhancement of ROMK1 channel activity by LEV

ROMK1 channels are sensitive to intracellular protons with an effective pK_a value of ~6.9 [18,20]. We examined whether changes in the pH_i affects the activity of wild-type ROMK1 channels by LEV. In pH_i 7.0, LEV (0.5 and 1 mM) caused a significant increase in wild-type ROMK1 channel activity by (% of control) 5.86 ± 0.29 and 10.56 ± 1.02 ($n = 10$, $P < 0.05$, Fig. 2A). Similar results are also found in pH_i 9.4, LEV (0.5 and 1 mM) enhanced ROMK1 channel activity by (% of control) 6.23 ± 0.35 and 11.67 ± 0.51 ($n = 10$, $P < 0.05$, Fig. 2B). As comparing the enhancement effects of LEV in pH_i 7.0 and 9.4, there was no significant difference in its activation of ROMK1 channels at different pH_i values ($P > 0.05$, Fig. 2C).

The amino acid responsible for the pH_i sensitivity of ROMK has been identified as Lys-80 in the N-terminus. Substitution of Lys-80 by methionine abolishes the sensitivity of ROMK1 to intracellular protons [18]. LEV (0.5 mM) had a similar significant potentiation effect on K80M channel activity (% of control) in pH_i 9.4 (6.05 ± 0.62), pH_i 7.4 (5.15 ± 1.36), or pH_i 6.9 (3.91 ± 0.22, $n = 5$, $P < 0.05$, Fig. 2D–F). Similar results were also found in the higher dose of LEV (1.0 mM) on the enhancement of K80M channel activity (% of control) in pH_i 9.4 (11.02 ± 0.97), pH_i 7.4 (10.35 ± 1.45), or pH_i 6.9 (10.18 ± 0.31, $n = 5$, $P < 0.05$, Fig. 2D–F).

3.3. Effect of PIP_2 on the enhancement of ROMK1 channel activity by LEV

A characteristic feature of all Kir channels is regulated by PIP_2 [19]. The mediated residues involved in this interaction of ROMK1 channels and PIP_2 are the amino acid Arg-188, Arg-217 and Lys-218 [19,25]. After administering LEV (0.5 and 1 mM) to

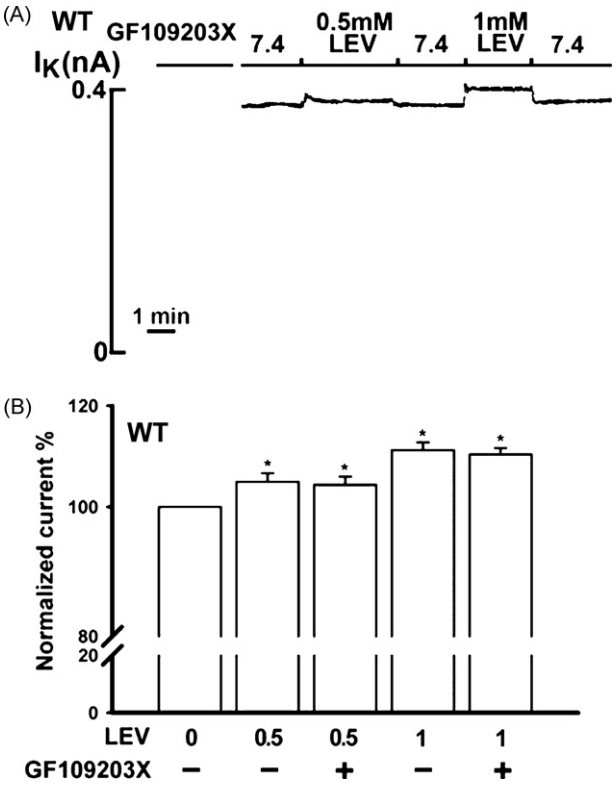

Fig. 4 – LEV activates ROMK1 channels may not through PKC-dependent pathway. (A) ROMK1 channels expressed in oocytes were pre-incubated with GF109203X (10 μM) for 5 min then performed the inside-out patch recordings. LEV (0.5 and 1 mM) activates ROMK1 channel activity in these preparations. (B) The percentage of the activation effect of LEV (0.5 and 1 mM) on ROMK1 channels by application of LEV and PKC inhibitors (GF109203X "+", $n = 6$ for each group). * indicates $P < 0.05$ by ANOVA.

three PIP_2-binding sites site-mutated channels (R188Q, R217A and K218A), the activity of channels was enhanced by (% of control) 5.07 ± 0.64 and 10.02 ± 1.35 in R188Q (Fig. 3A), 4.88 ± 0.98 and 11.14 ± 1.28 in R217A (Fig. 3B), or 4.34 ± 0.73 and 10.26 ± 1.03 in K218A ($n = 6$ in each group, $P < 0.05$, Fig. 3C), these effects being similar to those of LEV on the wild-type ROMK1 channels.

3.4. *Effect of PKC on the enhancement of ROMK1 channel activity by LEV*

ROMK1 channels have been reported to inhibit by PKC [26]. After pre-incubation with PKC specific inhibitor GF109203X (10 μM) for 5 min. LEV (0.5 and 1 mM) enhanced ROMK1 channel activity by (% of control) 3.45 ± 1.24 and 10.21 ± 0.99 ($n = 6$, $P < 0.05$, Fig. 4).

ROMK1 channels have one putative PKC-phosphorylation site located in the N-terminus (Ser-4) and five putative PKC-phosphorylation sites at the C-terminus (Ser-183, Thr-191, Thr-193, Ser201 and Thr-234) [27]. Treatment of LEV (0.5 and 1 mM) enhanced mutants for these PKC-phosphorylation sites mutated channels by (% of control) 3.15 ± 0.51 and 7.17 ± 1.03 in S4A, 3.45 ± 0.84 and 6.21 ± 0.38 in S183A, 4.19 ± 0.43 and 10.62 ± 1.63 in T191A, 4.91 ± 0.65 and 10.67 ± 0.77 in T193A, 3.78 ± 0.40 and 6.46 ± 0.69 in S201A or 5.16 ± 0.65 and 7.19 ± 0.65 in T234A ($n = 6$, $P < 0.05$, Fig. 5).

3.5. *Effect of PKA on the enhancement of ROMK1 channel activity by LEV*

ROMK1 channels are regulated by PKA via direct phosphorylation of the channels [28]. Pre-incubation with PKA specific inhibitor H89 (10 μM) and KT5720 (2 μM) for 5 min, LEV (1 mM) failed to activate ROMK1 channel activity ($0.21 \pm 0.95\%$ and $0.04 \pm 1.01\%$ of control, $n = 6$, $P > 0.05$, Fig. 6).

Previous report has indicated that Ser-44 on the N-terminus, Ser-219 and Ser-313 on the C-terminus are PKA phosphorylated sites of ROMK1 channels [28]. Application of

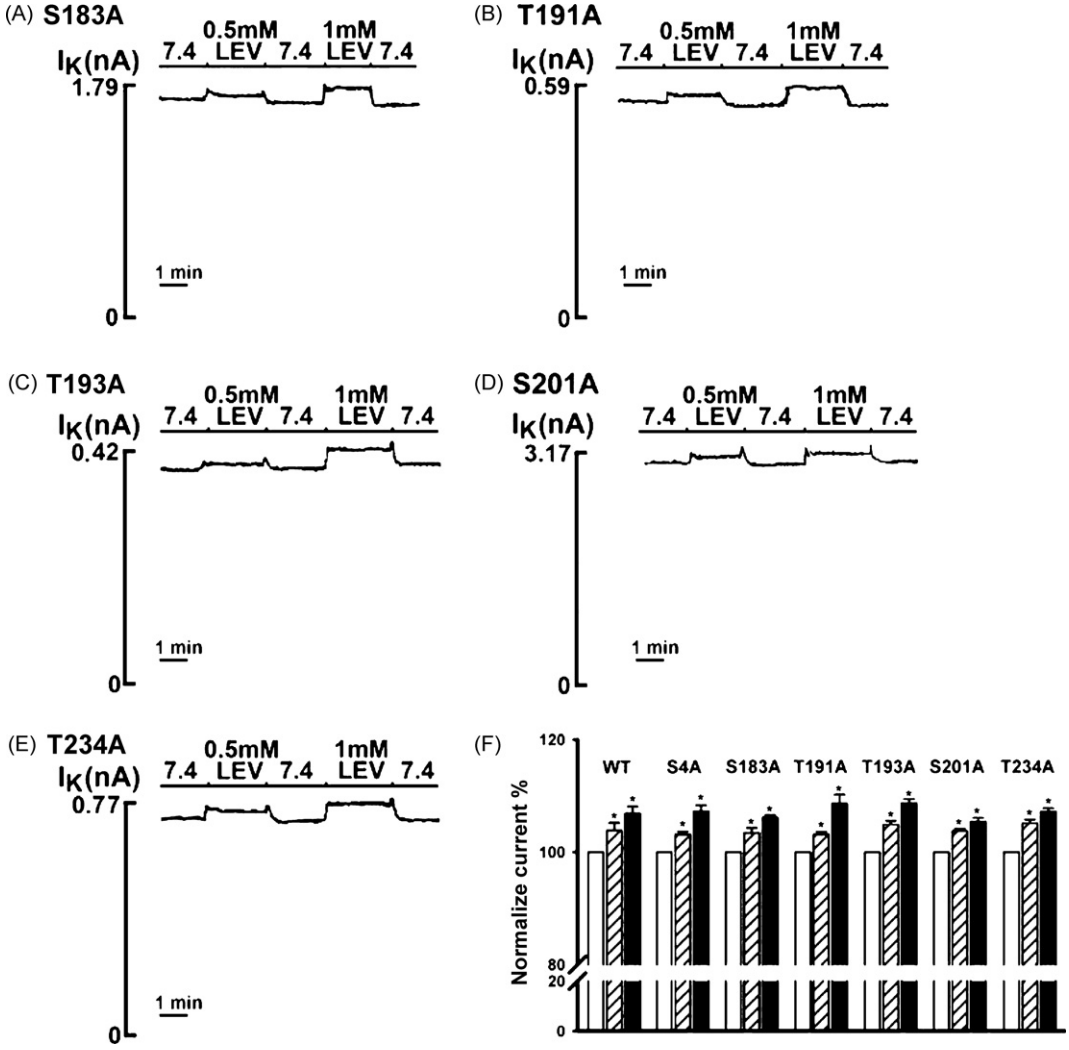

Fig. 5 – LEV activates PKC-phosphorylation sites mutated ROMK1 channels currents. (A)–(E) LEV (0.5 and 1 mM) activates five PKC putative phosphorylation sites mutants (S183A, T191A, T193A, S201A and T234A). (F) The percentage of the activation effect of LEV on the wild-type (WT) and these mutant channels of ROMK1 channels ($n = 6$ for each group). * indicates $P < 0.05$ by ANOVA. Blank columns represent the control group without LEV. Hatch columns represent that channels were treated with 0.5 mM LEV. Black columns represent that channels were treated with 1 mM LEV.

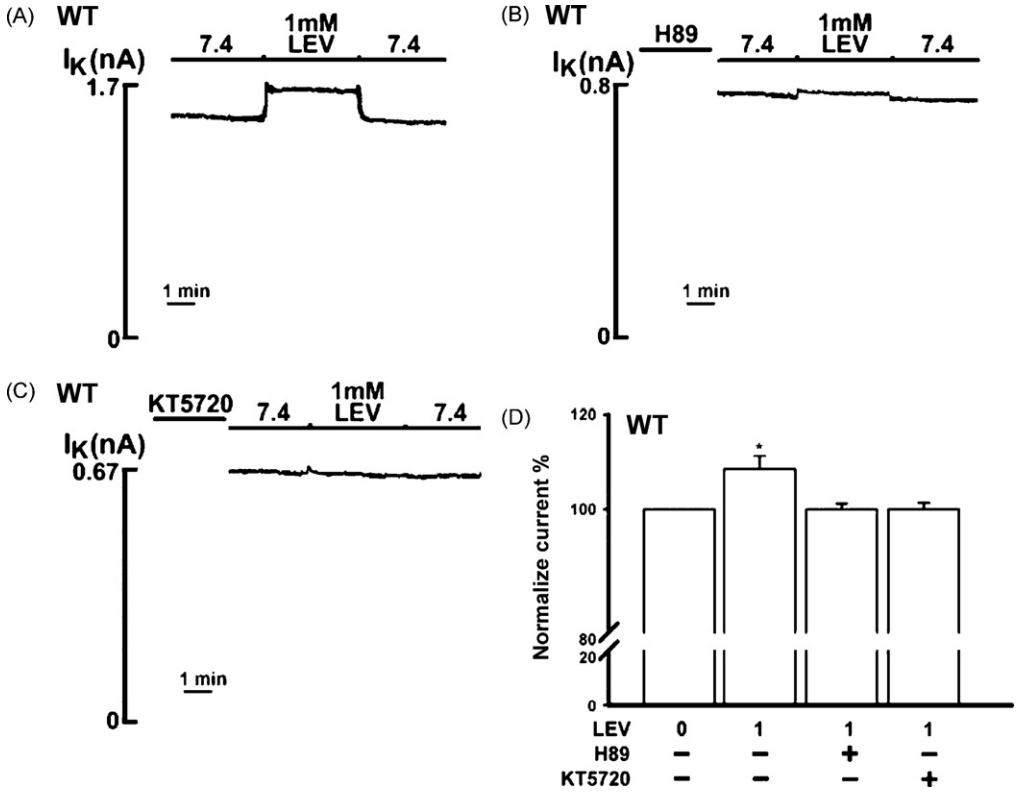

Fig. 6 – LEV enhances ROMK1 channel activity rely on a PKA-dependent pathway. (A) LEV (1 mM) activates the wild-type (WT) ROMK1 currents. (B) ROMK1 channels expressed in oocytes were pre-incubated with H89 (10 μM) and (C) KT5720 (2 μM) for 5 min then performed the inside-out patch recordings. LEV (1 mM) failed to enhance the ROMK1 channels activities that have been treated with PKA inhibitors. (D) The percentage of the activation effect of LEV (1 mM) on ROMK1 channels by application PKA inhibitors (H89 and KT5720, $n = 4–8$ for each group). * indicates $P < 0.05$ by ANOVA. "+" indicates in the presence of H89 or KT5720.

LEV (1 mM) was observed to enhance the S44A mutant channels ($8.18 \pm 1.13\%$ of control, $n = 8$, $P < 0.05$, Fig. 7A). However, LEV has no potentiation effect on the S219A and S313A mutant channels ($0.11 \pm 1.08\%$ and $0.04 \pm 1.15\%$ of control, $n = 8$, $P > 0.05$, Fig. 7B and C).

PKA mediates the activity of ion channels by a variety of mechanisms, for example, inducing a conformational change and/or addition of negative charges [21]. Mutation of the PKA target sites Ser-44, Ser-219 or Ser-313 to aspartate, which mimic the negative charge carried by a phosphate group bound to a serine. Although LEV (1 mM) enhanced the S44D mutant ($8.78 \pm 1.09\%$ of control, $n = 6$, $P < 0.05$, Fig. 8A), LEV has no potentiation effect on the S219D and S313D mutant channels ($0.02 \pm 0.95\%$ and $0.04 \pm 1.01\%$ of control, $n = 6$, $P > 0.05$, Fig. 8B and C). In addition, LEV (1 mM) did not enhance the S219R mutant, in which Ser-219 is replaced by the positive charged of arginine ($0.21 \pm 0.45\%$ of control, $n = 6$, $P > 0.05$, Fig. 8D). These results indicated that the effect of LEV may not come from the charge–charge interaction in the ROMK1 channels.

4. Discussion

The present study demonstrated that LEV increased ROMK1 channel activity in a concentration-dependent manner. ROMK1 channels are regulated by multiple signaling pathways, including membrane PIP_2, pH_i, PKC and PKA. This drug increases both pH_i gating residue mutants and wild-type channels activity over a range of pH_i values. The effect of LEV is independent of the intracellular protons. In addition, LEV enhances the PIP_2-binding residues mutant channels activity, suggesting that LEV activate ROMK1 channels is not through PIP_2 pathway. Similar enhancement effects of LEV were observed in the presence of PKC inhibitor as well as in the PKC-mediated phosphorylation sites mutant channel, showing that its effects were not mediated via the PKC pathway. Since LEV fails to enhance the ROMK1 channel activity in the presence of PKA inhibitors, LEV potentiates the activity of the channels through a PKA-dependent mechanism. This observation is further supported by the evidence that LEV has no effect on the PKA-phosphorylation sites mutated channels (S219A and S313A).

Phosphorylation of channels by PKA is important for the sensitivity of AEDs. Topiramate has been demonstrated to bind to the PKA-phosphorylation sites of AMPA/kainite receptors to control channel conductance by an allosteric modulatory effect [29]. PKA potentiates the inhibitory effect of gabapentin on voltage-activated Ca^{2+} channels [30]. The PKA-phosphorylation sites of ROMK1 channels on the N- and C-terminal regions modulate channel activity by different

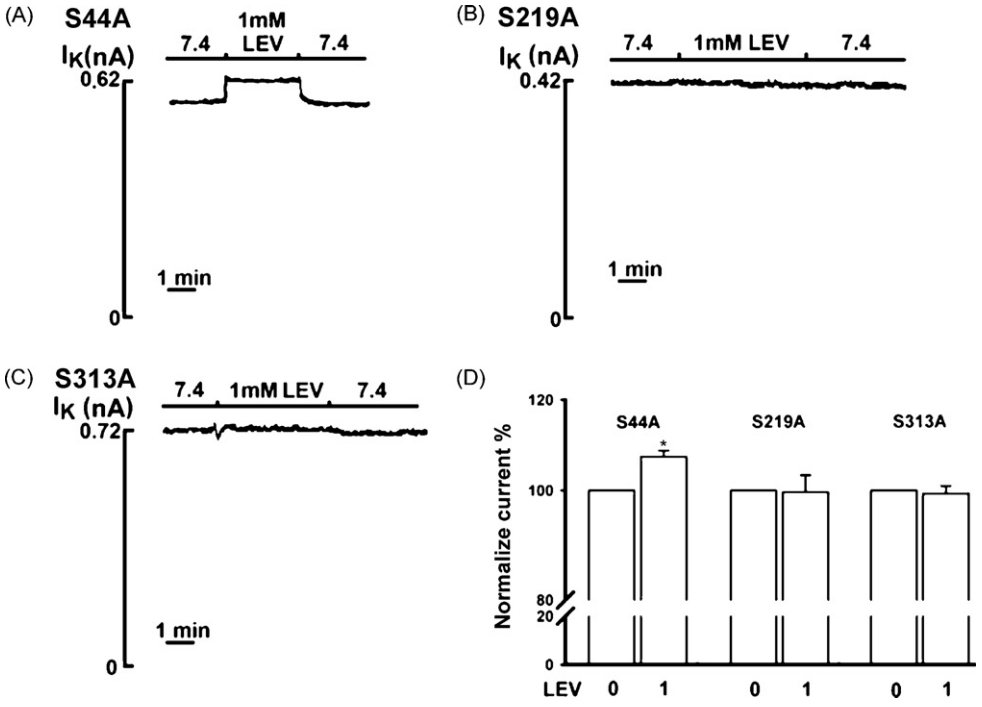

Fig. 7 – Effects of LEV on the PKA-phosphorylation sites mutated ROMK1 channels. (A) LEV (1 mM) enhances the activity of S44A channels, which is the PKA-phosphorylation site-mutated ROMK1 channel located in N-terminus. (B) LEV has no effect to activate S219A and (C) S313A channels. These two channels are the PKA-phosphorylation sites mutated ROMK1 channel located in C-terminus. (D) The percentage of the activation effect of LEV (1 mM) on S44A, S219A and S313A channels ($n = 8$ for each group). * indicates $P < 0.05$ by ANOVA.

mechanisms. The phosphorylation sites at the C-terminal S219 and S313 control the open probability of the channel by regulating the stability of the open state [31]. The PKA-phosphorylation site at the N-terminal S44 determines the number of conducting channels by masking endoplasmic reticulum (ER) retention signals and then stimulating the forward trafficking and surface delivery of ROMK1 channels [32]. In this study, we found that LEV enhances activity of the S44A mutant as the wild-type ROMK1 channels. These findings suggest that the effect of LEV is not caused by the phosphorylation induced ER export. However, LEV has no potentiation effect on the S219A and S313A mutant channels. PKA-mediated phosphorylation for stabilizing the open state of ROMK1 channels is important for the activation effects of LEV to the channels.

In addition, LEV cannot enhance the activity of constructed mutants (S219D and S313D) that mimic the negative charge carried by a phosphate group bound to a serine nor a mutated channel with an additional positive charge (S219R). It is indicated that the PKA-mediated phosphorylation effect for LEV on the ROMK1 channels may not come from the charge–charge interaction. The PKA-mediated phosphorylation induced conformational changes on ROMK1 channels may contribute to the mechanisms of LEV.

The molecular mechanisms underlying the therapeutic effects of the second generation AEDs have been attributed to modulating the function of Kir channels and voltage-gated K^+ channels. Retigabine stabilizes the open-pore conformation of KCNQ channels by binding to the channel's activation gate between the cytoplasmic parts [33]. Lamotrigine enhances transient K^+ outward current in CA1 pyramidal cells and in neocortical cells [34] and inhibit A-type K^+ current in hippocampal neuron [35]. Gabapentin inhibits K^+-evoked noradrenaline release through activation of K_{ATP} channels [36] and activates $GABA_B$ receptor to induce Kir3 currents resulting in reduced hippocampal epileptiform discharges [37]. Pregabalin activates K_{ATP} channels in hippocampal neurons by increasing the open probability of the K_{ATP} channels [38]. LEV decreases voltage-gated K^+ channels to depress the effect on generation of action potential [6]. Since ROMK1 channels have been reported to maintain the RMP during the cellular excitation in the hippocampal neurons [17], activation of ROMK1 channels to reduce neuronal excitability may be an important mechanism for the antiepileptic effect of LEV.

LEV significant increases ROMK1 currents in the range of therapeutic concentration (100 μM or 250 μM) in spite of its EC_{50} (363 μM) value are higher than that for seizure control [6]. Our results revealed that the effect of LEV on ROMK1 channels is small. Kir channels are the major determinant for RMP. Slightly changes of Kir currents can induce significant alterations of RMP which may cause large membrane depolarizations or hyperpolarizations [39]. Open small amount of ROMK1 channels have been reported to decrease membrane potential near RMP resulted in neuron silencing, while higher amounts lead to neurons that cannot fire in the

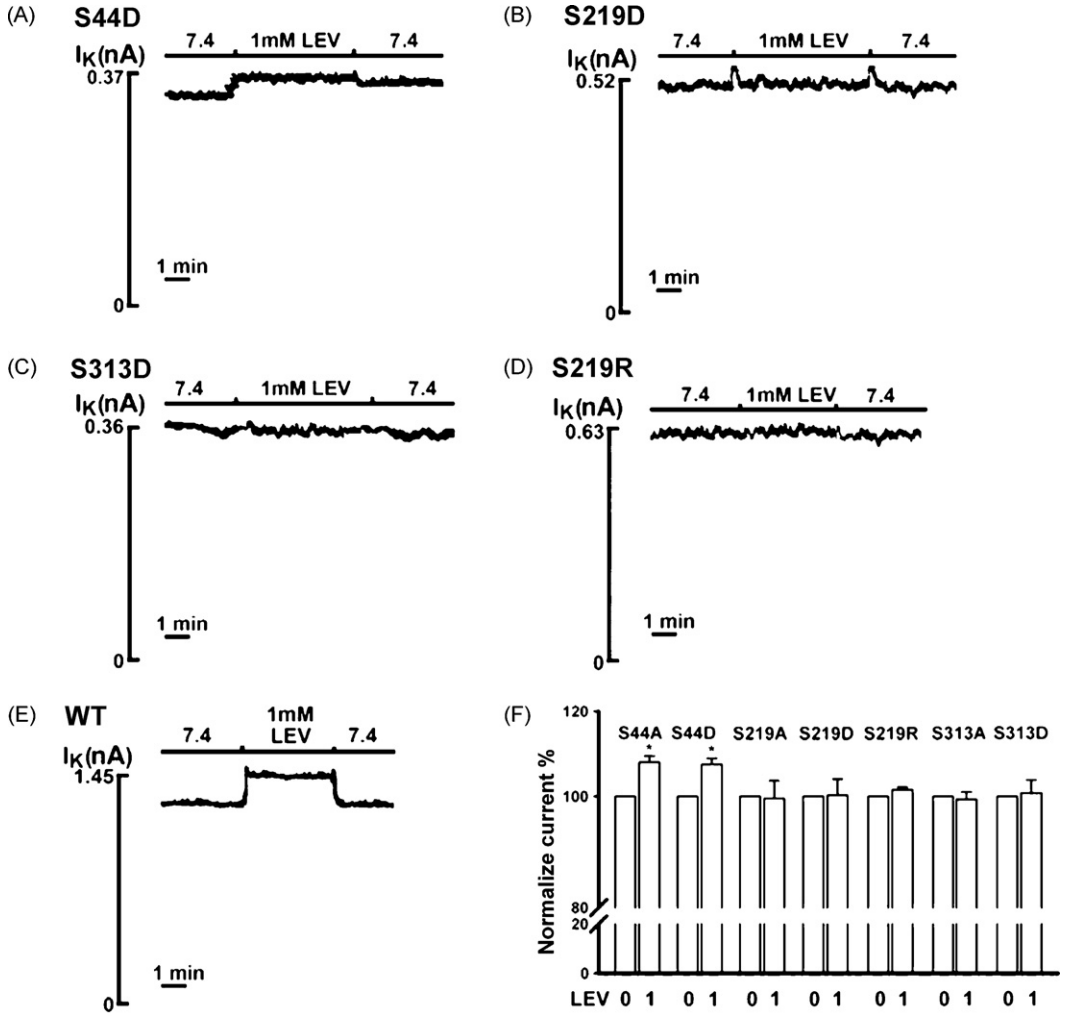

Fig. 8 – The charge effect of PKA-mediate phosphorylation for LEV functions in ROMK1 channels. The mutation of PKA-phosphorylation sites to aspartate (S44D, S219D and S313D) which mimic the negative charge carried by a phosphate group bound to a serine. (A) LEV (1 mM) enhance on S44D mutated channels. (B) LEV (1 mM) has not enhancement effects on S219D and (C) S313D mutated channels. (D) The mutants activity Ser-219 replaced by arginine (S219R), a positive charge, substitutes the effect of LEV (1 mM) in activation channel currents. (E) An application of LEV (1 mM) activates the wild-type ROMK1 currents (WT). (F) The percentage of the activation effect of LEV (1 mM) for ROMK1 channels by the mutant of ROMK1 channels (S44A, S44D, S219A, S219D, S219R, S313A and S313D, $n = 6$ for each group) * indicates $P < 0.05$ by ANOVA.

hippocampal neurons [19]. Our results revealed the maximum enhancement effect of LEV is 12% on ROMK1 currents, it is important for the maintenance of RMP during neuronal excitation.

In this study, LEV is dissolved in the FVPP solution which contains HEPES as the buffer system. It will maintain a stable pH_i value in our experiment. We found LEV potentiates ROMK1 channel activity at both high pH_i (9.4) and low pH_i (7.0) values. Although LEV has been reported to lower pH_i in neurons [40], our results revealed LEV can potentiate ROMK1 channels in the condition of low pH_i.

Docking and fusion between transport vesicles and target acceptor membranes involve the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins such as SV2, syntaxins, synaptobrevins, synaptotagmins, synaptophysins and SNAP-25 [41]. The identification SV2A as the binding site in brain for LEV has important implications for the antiepileptic action of LEV. It is possible that LEV-binding enhances a function of SV2A that inhibits abnormal bursting in epileptic circuits [42]. In addition, SNARE proteins play important roles in directly regulating the function of the ion channels [43]. Syntaxin 1A has been reported to inhibit ROMK1 channel activity and that synaptobrevin 2 reverses the inhibition [44]. The effect of LEV in ROMK1 channels related to the SNARE proteins should be an interesting subject for further investigations.

In conclusion, the function of LEV on the ROMK1 channels is independently of which mechanisms relate to the pH_i, PIP_2, and PKC. It depends on a novel PKA-mediated phosphorylation pathway which may induce conformational change on the channels. The effect of LEV in enhancing the activity of ROMK1 channels and restore neuronal RMP to prevent seizure

spreading may play an important role in its antiepileptic effects.

Disclosure of interest

We declare that, except for income received from our primary employer, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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