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Original Article

Dual regulatory effects of PI(4,5)P_2 on TREK-2 K^+ channel through antagonizing interaction between the alkaline residues (K^{330} and $R^{355-357}$) in the cytosolic C-terminal helix

Sung Eun Kim[1], Myoung-Hwan Kim[1], Joohan Woo[2,3,*], and Sung Joon Kim[1,4,*]

[1]Department of Physiology, Seoul National University College of Medicine, [2]Department of Physiology, Dongguk University College of Medicine, Gyeongju 38066, [3]Channelopathy Research Center (CRC), Dongguk University College of Medicine, Goyang 10326, [4]Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine, Seoul 03080, Korea

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***Correspondence**
Sung Joon Kim
E-mail: sjoonkim@snu.ac.kr
Joohan Woo
E-mail: lsjoohan@gmail.com

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ABSTRACT TWIK-related two-pore domain K^+ channel-2 (TREK-2) has voltage-independent activity and shows additional activation by acidic intracellular pH (pH_i) *via* neutralizing the E^{332} in the cytoplasmic C terminal (Ct). We reported opposite regulations of TREK-2 by phosphatidylinositol 4,5-bisphosphate (PIP_2) *via* the alkaline K^{330} and triple Arg residues ($R^{355-357}$); inhibition and activation, respectively. The G^{334} between them appeared critical because its mutation ($G^{334}A$) endowed hTREK-2 with tonic activity, similar to the mutation of the inhibitory K^{330} ($K^{330}A$). To further elucidate the role of putative bent conformation at G^{334}, we compared the dual mutation forms, $K^{330}A/G^{334}A$ and $G^{334}A/R^{355-7}A$, showing higher and lower basal activity, respectively. The results suggested that the tonic activity of $G^{334}A$ owes to a dominant influence from R^{355-7}. Since there are additional triple Arg residues (R^{377-9}) distal to R^{355-7}, we also examined the triple mutant ($G^{334}A/R^{355-7}A/R^{377-9}A$) that showed tonic inhibition same with $G^{334}A/R^{355-7}A$. Despite the state of tonic inhibition, the activation by acidic pH_i was preserved in both $G^{334}A/R^{355-7}A$ and $G^{334}A/R^{355-7}A/R^{377-9}A$, similar to the $R^{355-7}A$. Also, the inhibitory effect of ATP could be commonly demonstrated under the activation by acidic pH_i in $R^{355-7}A$, $G^{334}A/R^{355-7}A$, and $G^{334}A/R^{355-7}A/R^{377-9}A$. These results suggest that the putative bent conformation at G^{334} is important to set the tug-of-war between K^{330} and R^{355-7} in the PIP_2-dependent regulation of TREK-2.

INTRODUCTION

Two-pore domain K^+ (K2P) channel family proteins coded by *KCNK* genes assemble as dimers to form a functional K^+ channel. Each subunit has four transmembrane domains (TM1–TM4), two-pore domains (P1 and P2), and intracellular N- and C-termini. The K2P channels show voltage-independent activity, consistent with the "leak-type" background K^+ conductance observed in various types of cells [1-3]. However, some members of the K2P channels show wide ranges of regulation by cellular signals and by various conditions. Especially, TWIK-related K^+ channels (TREKs) such as TREK-1 (KCNK2) and TREK-2 (KCNK10) show sensitivity to physicochemical stimuli; activation by membrane stretch, polyunsaturated fatty acid, and acidic intracellular pH (pH_i) [4-7]. Different from the other members of K2P channels, the TREKs show relatively low basal activity that is largely increased by the various kinds of stimuli. As for the low basal activity, tonic inhibition by the physiological level of phosphatidylinositol 4,5-bisphosphate (PIP_2) has been suggested as the key mechanism including our previous studies (Fig. 1A) [8-14].

In general, basic amino acids such as Arg (R) and Lys (K) in the cytoplasmic domain of ion channels are responsible for the PIP_2-

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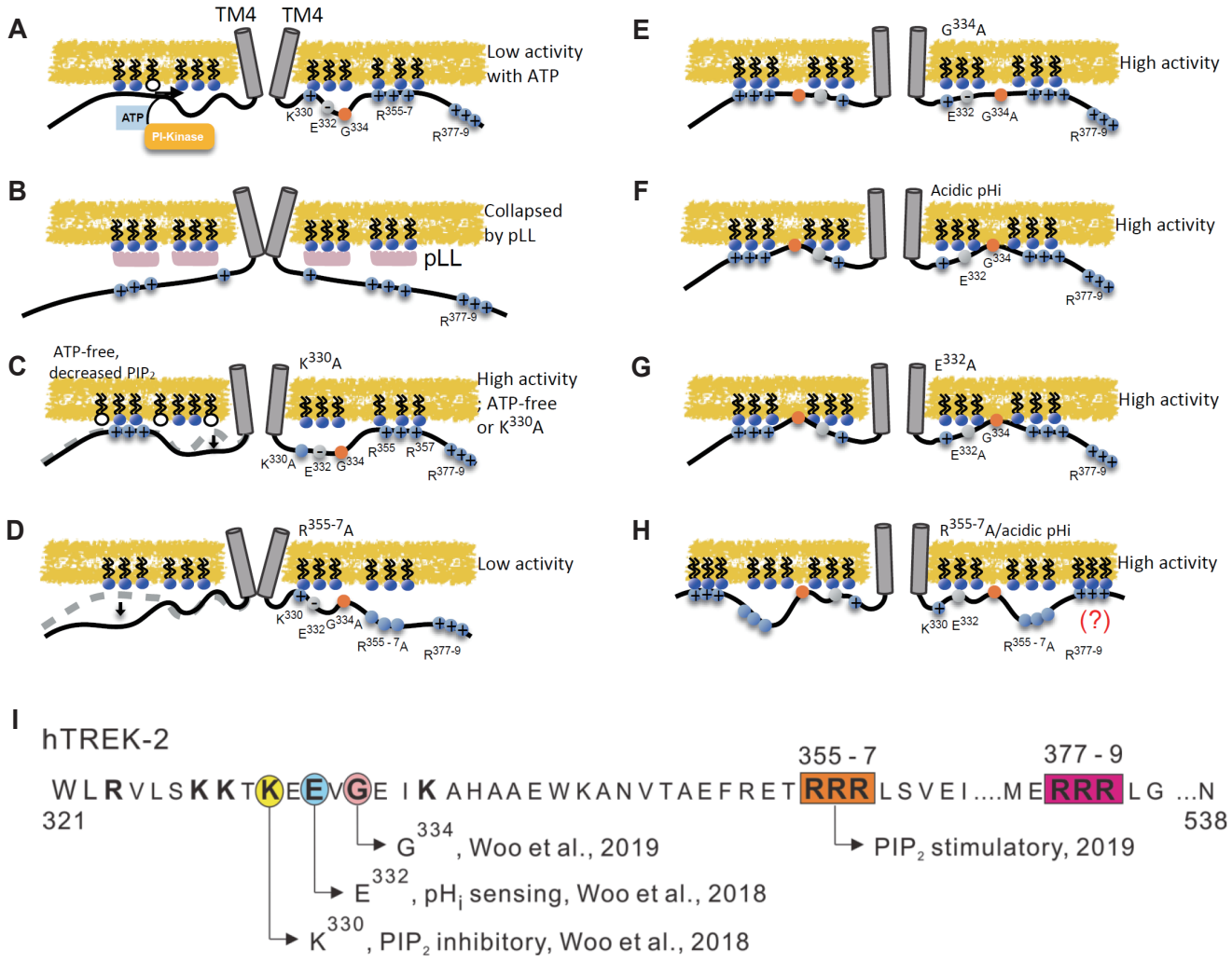

Fig. 1. The proposed mechanisms of regulatory interactions between Ct and membrane PIP_2 depicted as cartoon models. Schematic drawing of hTREK-2 dimers with TM4 connected to proximal C-terminus (Blue and grey circles for that cationic and anionic residues. Orange circles for the G^{334}). (A) Low activity of hTREK-2 with increased PIP_2 level. (B) Applied PIP_2 scavenger (pLL) drives hTREK-2 into the inhibited state. (C) High activity state with ATP-free or neutralization of Lys ($K^{330}A$). (D) Soley mutated of Arg ($R^{355-7}A$) induces low activity. (E) High activity state with mutated G^{334}, decreased hinge flexibility. (F, G) hTREK-2 is highly activated by acidic pH_i and neutralizing mutation, $E^{332}A$. (H) $R^{355-7}A$ mutation showed high activity in acid pH_i. Distal triple cationic residues (R^{377-9}) function were still unknown. (I) The sequence of Ct amino acids in hTREK-2. A pink circle indicates G^{334} which roles of putative hinge residue. Orange and Red boxes indicate consecutive proximal and distal cationic residues; R^{355-7} and R^{377-9}. Yellow and blue circles indicate PIP_2-dependent inhibitory and pH_i sensing site; K^{330} and E^{332}.

dependent regulation via electrostatic binding with the anionic head groups of the phospholipids. The direction of regulation, activation or inhibition, appears controversial and diverse depending on the channels. Regarding the TREKs, their complete inhibition by cytoplasmic-side application of the polycationic PIP_2 scavenger (e.g., poly-L-lysine [pLL]) initially indicated a simple stimulatory role of PIP_2 (Fig. 1B) [9]. However, further investigations by us have shown more complex regulatory features; the inhibition by a relatively high level of PIP_2 and the activation by a partial decrease of PIP_2. The latter type of activation is demonstrated by the removal of cytoplasmic ATP that results in the spontaneous decrease of PIP_2 by lipid phosphatase. Vice versa, the tonic inhibition by the physiological level of PIP_2 maintained by the membrane-associated phosphoinositide (PI)-kinase and ATP [13] (Fig. 1A).

The dual-mode of TREK channel regulation by PIP_2 appears to be concentration-dependent, and the site-directed mutagenesis studies of the proximal cytoplasmic C-terminal region (pCt) could elucidate the corresponding sites [13-15]. In terms of the human TREK-2 (hTREK-2) sequence, the Lys (K^{330}) in the early pCt is responsible for the inhibitory regulation by a relatively higher level of PIP_2 (Fig. 1C) [14]. In contrast, three consecutive Arg residues (R^{355-7}) mediate the positive regulation by PIP_2 (Fig. 1D) [15]. Although there are additional triple Arg residues (R^{377-9}) distal to the R^{355-7}, their mutation by Ala substitution ($R^{377-9}A$) did not affect the activity of TREK-2 [15]. Therefore, it was assumed

that the more distal R^{377-9} residues are not contributing to the regulation by PIP_2, or might not be bound to the negatively charged phospholipids (Fig. 1A).

In addition to the mutually antagonizing roles of K^{330} and R^{355-7}, an interesting role of Gly (G^{334}) between them was suggested from our recent study; its mutation by Ala substitution ($G^{334}A$) converts into a tonic active state, similar to the $K^{330}A$ (Fig. 1E) [15]. Because Gly could confer flexibility or bent conformation to the protein structure, the drastic changes of channel activity in $G^{334}A$ might reflect the outcome of a 'tug of war' between K^{330} and R^{355-7}, i.e., dominating influence of the stimulatory R^{355-7} and putative loss of the electrostatic binding between K^{330} and PIP_2 in $G^{334}A$. However, such speculation could not exclude the possibility that the G^{334} mutation might have changed the channel property independent of the mutual interaction between K^{330} and R^{355-7}.

Another key feature of TREK-2 is the activation by acidic pH_i that is mediated by the titration of the negatively charged Glu (E^{332}) close to the inhibitory K^{330} [10,11,13,14]. Our hypothetical model of the pH_i-dependence is that the inhibitory influence from K^{330} could be relieved by the neutralization of E^{332} (Fig. 1F, G). In fact, the tonic high activity in $K^{330}A$ was not further activated by acidic pH_i [14]. In the activation by acidic pH_i, the putatively bent conformation at the nearly Gly residue (G^{334}) appears critical because its mutation ($G^{334}A$) also abolished the activation by acidic pH_i as well as inducing tonic high activity (Fig. 1E) [15]. An interesting finding in our previous study was that the activation by acidic pH_i could be still induced in $R^{377-9}A$ [15]. The result suggested a possibility that R^{377-9} might play a hidden role as the putative assistant binding with PIP_2 (Fig. 1H), which was not investigated.

With this background, in the inside-out patch-clamp studies of the hTREK-2 and its various mutants, we compared their sensitivity to ATP and acidic pH_i to get the clues to further understand the structure-function relation of TREK-2. The specific question was whether the combined mutation of G^{334} with K^{330} ($K^{330}A/G^{334}A$) or with R^{355-7} ($G^{334}A/R^{355-7}A$) show similar or different properties with the previous findings in $K^{330}A$, $G^{334}A$, and $R^{355-7}A$. We hypothesized that if the putative bent conformation in the G^{334} of wild-type (WT) hTREK-2 simply mediates the tug-of-war between K^{330} and R^{355-7}, the properties of double mutants, i.e., $K^{330}A/G^{334}A$ and $G^{334}A/R^{355-7}A$, would be the same with the properties of $K^{330}A$ and $R^{355-7}A$, respectively.

Apart from this question, we also asked whether the combined triple mutations of R^{355-7} and R^{377-9} with G^{334} ($G^{334}A/R^{355-7}A/R^{377-9}A$) show similar or different properties with double mutants in G^{334} and R^{355-7} ($G^{334}A/R^{355-7}A$). The experimental result might elucidate a hypothetical hidden role of R^{377-9} in hTREK-2.

METHODS

Cell culture and preparation

Human embryonic kidney (HEK293T) cells were purchased from ATCC (Manassas, VA, USA) and incubated in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% (50/500 ml) fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin (Gibco) at 37°C in 20% O_2– 5% CO_2.

Heterologous expression of hTREK-2

The human complementary DNAs (cDNAs) of TREK-2 (hTREK-2, SC222775) was purchased from ORIGENE and was transfected into HEK293T cells using a TurboFect transfection reagent (ThermoFisher Scientific, Waltham, MA, USA). Point mutations in hTREK-2 were generated using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Palo Alto, CA, USA). Sequences of the mutants were confirmed by DNA sequencing.

Electrophysiology

Cells were transferred into a bath mounted on the stage of an inverted microscope (TE2000-S; Nikon, Tokyo, Japan). The bath (0.15 ml) was perfused at 5 ml/min, and voltage-clamp experiments were performed at room temperature (22°C–25°C). Patch pipettes with a free-tip resistance of about 3–4 MΩ were used for inside-out (i-o) patch-clamp. The pipettes were connected to the head stage of a patch-clamp amplifier (Axopatch-1C; Axon Instruments, Foster City, CA, USA). pCLAMP software v.10.6 and Digidata-1322A (Axon Instruments) were used to acquire data and apply command pulses. The recorded currents were sampled at 10 kHz and were low-pass filtered at 2 kHz (i-o). The stored currents were analyzed using Clampfit v. 10.6 and Origin v.8.0 (Microcal, Northampton, MA, USA).

Solutions and chemicals

Both pipette solution and bath solutions of i-o patch-clamp contained (in mM) 145 KCl, 0.1 EGTA, 10 HEPES, and 20 sucrose with a pH of 7.0 (titrated with KOH). For acidic (pH 6.0, pH 5.0) solution applied to the cytoplasmic side of i-o patch-clamp, half of HEPES was substituted with 5 mM MES (2-[*N*-morpholino] ethanesulfonic acid). The chemicals and drugs used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical analysis

Data are represented as mean ± standard error of the mean (SEM). Results were compared using paired or unpaired two-

sample t-test was employed for comparison of data collected from mutations. p-values < 0.05 were considered statistically significant. In the figures, *, **, and *** indicate $p<0.05$, 0.01 and 0.001, respectively. All statistical analyses were done with Origin 2016 software (OriginLab, Northampton, MA, USA).

RESULTS

After making inside-out configuration of patch-clamp experiment, inward K^+ current was recorded at –60 mV of holding voltage with symmetrical 145 mM [K^+]. Since the activity of hTREK in the control state showed a tendency of spontaneous increase in the initial period after the membrane excision, we compared the responses to ATP and acidic pH_i at 2–3 min confirming a steady-state.

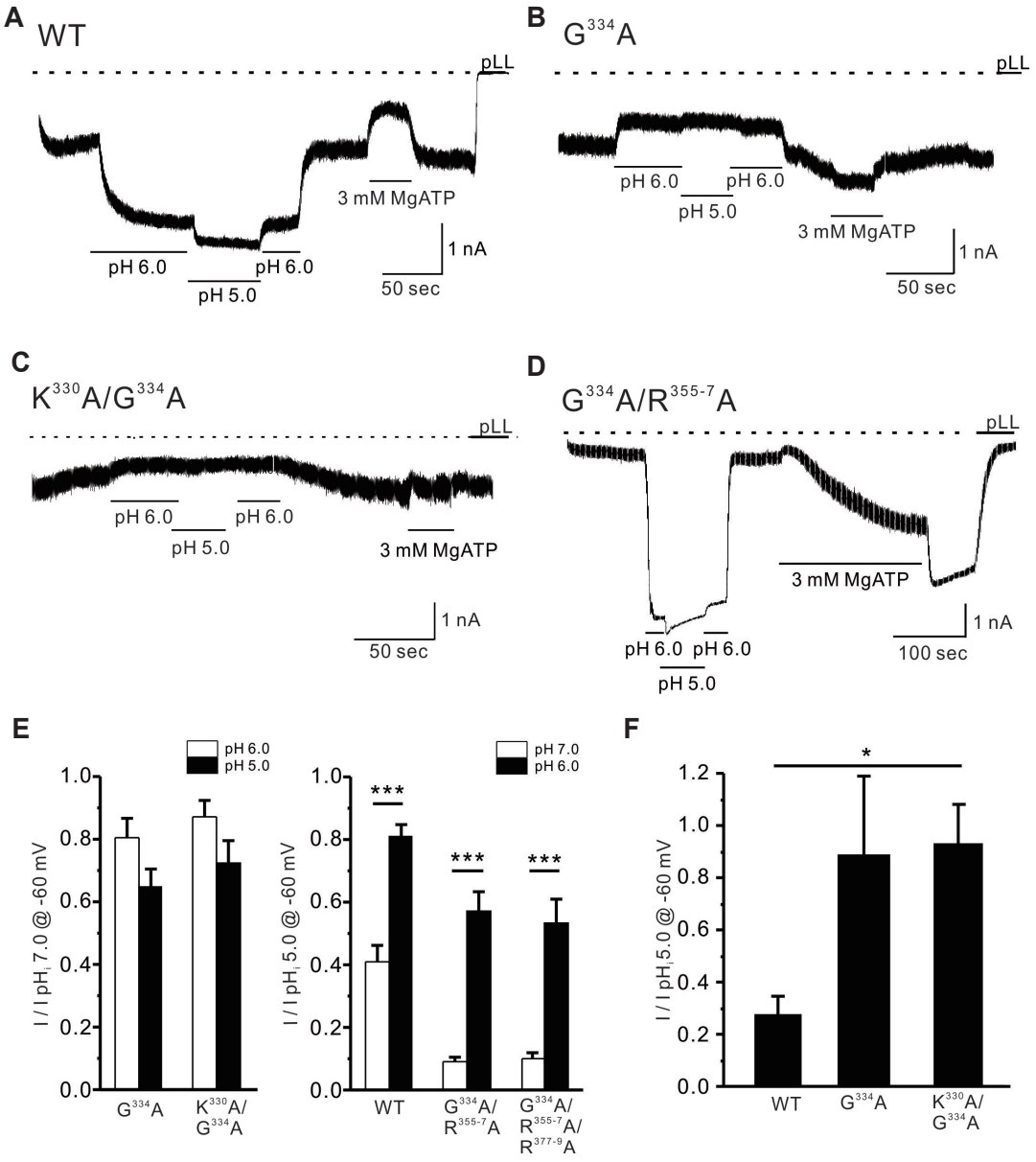

Fig. 2. Effects of acidic pH_i on the activity of hTREK-2 and its various mutations in Ct. Representative traces of inside-out patch clamp recording in wild-type (WT) (A), $G^{334}A$ (B), $K^{330}A/G^{334}A$ (C), $G^{334}A/R^{355-7}A$ (D). The current was measured at symmetrical 145 mM K^+ with –60 mV holding voltage. (A) Trace of WT showing spontaneous increase current after excised cell membrane. Activated by acidification (pH_i 6.0 and 5.0) and inhibited by 3 mM MgATP. (B, C) $G^{334}A$ and $G^{334}A/K^{330}A$ mutants high tonic activity, partial decrease of acid and slightly inhibition by 3 mM MgATP. (D) $G^{334}A/R^{355-7}A$ showed low basal activity, predominantly activated by acid and slowly activated by 3 mM MgATP. (E, F) Summary of currents at –60 mV normalized to the maximum level measured at pH_i 7.0 and pH_i 5.0. $G^{334}A$ (6), $K^{330}A/G^{334}A$ (13), WT (32), $G^{334}A/R^{355-7}A$ (28), $G^{334}A/R^{355-7}A/R^{377-9}A$ (16), respectively. *$p < 0.05$; ***$p < 0.005$.

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Representative traces of the inward K^+ currents from WT and the various mutants are shown in Fig. 2. The experimental protocols were, (1) changing pH_i from 7.0 to 6.0 and 5.0, (2) application of 3 mM MgATP to the cytoplasmic side at pH_i 7.0 or at the acidic pH_i depending on the mutant form, and (3) application of 10 μg/ml of pLL for the complete inhibition of TREK-2, confirming the baseline of recording.

The WT hTREK-2 showed prominent activation by pH_i 6.0 and further activation by pH_i 5.0. The application of MgATP induced reversible inhibition (Fig. 2A). In contrast, a large basal inward K^+ current (tonic activity) was observed in $G^{334}A$. Responses to acidic pH_i was the opposite of WT (Fig. 2B). Although not shown here, the application of MgATP at the neutral pH_i 7.0 induced no significant inhibition in $G^{334}A$ as demonstrated in our previous study [15].

Then, we investigated the properties of the newly induced mutants combined with $G^{334}A$. The $K^{330}A/G^{334}A$ showed a tonic activity that was not activated further but slightly inhibited by acidic pH_i. Also, the application of MgATP had no significant effect at pH_i 7.0. (Fig. 2C). The $G^{334}A/R^{355-7}A$ showed low basal activity and prominent activation by acidic pH_i. At pH_i 7.0, interestingly, the application of MgATP induced an initial inhibition followed by a slow activation that was not reversed but further enhanced by washout (Fig. 2D). The summary of the responses of the various hTREK-2 mutants to acidic pH_i is shown as the bar graphs (Fig. 2E). The difference in response to MgATP between mutants except $G^{334}A/R^{355-7}A$ was shown as a bar graph. The $G^{334}A/R^{355-7}A$ is discussed in Fig. 3 (Fig. 2F).

Finally, we investigated whether the additional mutations of the distal three consecutive Arg residues abolished the persistent activation by acidic pH_i in $G^{334}A/R^{355-7}A$. Different from the hypothesis, the property of $G^{334}A/R^{355-7}A/R^{377-9}A$ was not distinguishable from $G^{334}A/R^{355-7}A$; low basal activity, prominent activation by acidic pH_i, initial inhibition, and subsequent slow activation by MgATP (Fig. 3A). To further analyze the biphasic responses, MgATP was also applied at pH_i 6.0 (data not shown), which revealed more prominent changes of the three phases; (1) initial inhibition, (2) slow activation, and (3) rebound-like activation by washout (Fig. 3B). The biphasic responses to MgATP were similarly observed when $R^{355-7}A$ were solely mutated (Fig. 3C). The summary of the responses to MgATP is shown as the bar graphs (Fig. 3D). Interestingly, there was a tendency of lower slow activation phase in $R^{355-7}A$ than $G^{334}A/R^{355-7}A$ and $G^{334}A/R^{355-7}A/R^{377-9}A$.

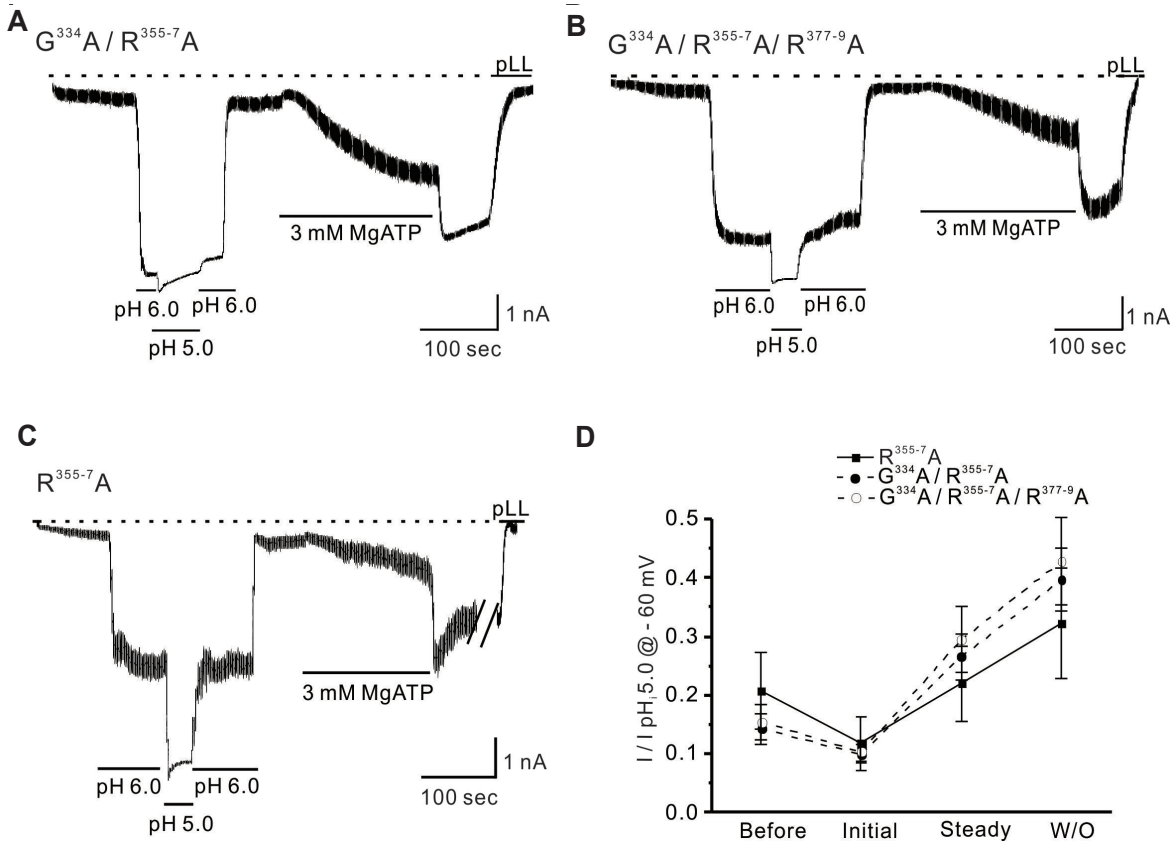

Fig. 3. Effects of intracellular ATP on the activity of hTREK-2 and its various mutations including the distal R^{377-9} in Ct. Representative traces of inside-out patch clamp recording in $G^{334}A/R^{355-7}A$ (A), $G^{334}A/R^{355-7}A/R^{377-9}A$ (B), and $R^{355-7}A$ (C). It has to be noted that the panel (A) is same with the trace shown in Fig. 2D. Symmetrical KCl condition with –60 mV of holding voltage was applied. After confirming the stimulatory effects of acidic pH_i, 3 mM MgATP was applied in pH_i 7.0. Initial current slightly inhibited by 3 mM MgATP then gradually increased (D). Summary of the TREK-2 currents at –60 mV normalized to the amplitude measured at pH_i 5.0. Mean ± SEM for $G^{334}A/R^{355-7}A$ (28), $G^{334}A/R^{355-7}A/R^{377-9}A$ (16), and $R^{355-7}A$ (10), respectively.

DISCUSSION

The relation between molecular structure and its function is an intriguing theme in a variety of proteins including ion channels. Among the amino acids comprising proteins, glycine (Gly) is the simplest amino acid having a single hydrogen atom as its side chain. The lack of a side chain makes Gly the most flexible amino acid. Due to its small and compact form, Gly is often integral to the packing formation of alpha-helices in the transmembrane domain proteins. Also, it facilitates helix kinking that increases the contact between helices [16]. Also, together with proline (Pro), Gly could provide structural flexibility of the protein [17].

Owing to the smallest size, changing Gly to other amino acids with a larger side chain could break the structure of proteins. For this reason, we tried to analyze the dual mutants. Our present study shows that the combined mutation of G^{334} with K^{330} or with R^{355-7} induces changes in the basal activity and the responses to the acidic pH_i and ATP, which were the same as the changed properties of $K^{330}A$ and $R^{355-7}A$. Therefore, it is not likely that the substitution of Gly with Ala ($G^{334}A$) extensively change the structure but only partly changed the balance between K^{330} and R^{355-7} with regard to the PIP_2-dependent regulation. Although the experimental findings are indirect and circumstantial, we cautiously interpret that the G^{334} residue could actually play a hinge- or fulcrum-like role between the inhibitory (K^{330}) and stimulatory (R^{355-7}) cationic residues in the Ct (Fig. 1).

The second question of this study was a suspicious hidden role of the more distal triple Arg (R^{377-9}) in the Ct of hTREK-2. Since both the $R^{355-7}A$ and the dual mutation ($G^{334}A/R^{355-7}A$) contained a similar capacity of activation by acidic pH_i, we supposed that the more distal R^{377-9} might provide another binding site with anionic phospholipids. However, our present study indicates that R^{377-9} may not play a significant role even after neutralizing the R^{377-9}. Therefore, the hypothesis of R^{377-9} as the second binding site was rejected. Nevertheless, since the relatively distal triple cationic residues are also conserved in the Ct of hTREK-1 (Fig. 1H), there might be still unknown regulatory function based on the electrostatic interaction, which requires future investigation.

An unexpected finding of the present study was the slow activation after the fast inhibition by ATP, a dual regulatory effect. The initial inhibition was consistent with our model of inhibitory interaction between PIP_2 and K^{330}, which has been consistently observed in the previous studies. However, there was no slow activation phase in the WT TREK-2 (Fig. 2A). But, slow activation phase was observed in $R^{355-7}A$, $G^{334}A/R^{355-7}A$, and $G^{334}A/R^{355-7}A/R^{377-9}A$ (Figs. 2, 3). Although statistically insignificant, the extent of slow activation appeared more prominent in the $G^{334}A/R^{355-7}A$ and $G^{334}A/R^{355-7}A/R^{377-9}A$ than $R^{355-7}A$ (Fig. 3D). In the experiment of the ATP effect, another notable finding was the rebound-like activation in response to the washout of ATP (Fig. 3). Together with the initial inhibition by ATP, it could be interpreted that the rebound-like activation might reflect the fast recovery from the inhibitory effect while the slower activation was not reversed yet. Taken together, the results suggested two independent mechanisms for the fast inhibition and slow activation by ATP in the proximal Arg (R^{355-7}) mutants. Although the PIP_2-dependent mechanism of the inhibitory effect is relatively well understood, the precise mechanism of slow activation is totally unknown yet. Nevertheless, since the slow activation was observed only in the site-directed artificial mutation of hTREK-2, the motivation for further investigation is not high.

The molecular structure of TREK-1 channel including the Ct was recently reported by Lolicato *et al.* [18]. The proximal region of Ct shows a long alpha-helix closed located to the inner leaflet of the plasma membrane. The putative bent conformation at Gly residue corresponding to G^{334} in hTREK-2 was not emphasized, and the possibility of Gly as the molecular fulcrum or hinge for the opposite regulation between K^{330} and R^{355-7} was not discussed in the study by Lolicato *et al.* [18]. According to our recent study and the present findings, the tonic high activity of $G^{334}A$ may be due to the putatively rigid conformation in the mutated alpha-helix of Ct. Based on the interpretation, we assumed that the flexibility or bent conformation at G^{334} may allow the K330-dependent inhibitory regulation with ATP in hTREK-2 (Fig. 1). Consistent with the hypothetical model, the combined mutation of $K^{330}A/G^{334}A$ showed a tonic active state as observed in the $K^{330}A$ of hTREK-2 (Fig. 2).

In summary, the present study of hTREK-2 and its various mutants could provide further, although indirect, evidence supporting our model of the dual regulation by PIP_2 *via* closely located cationic residues. The tug-of-war or see-saw model of Ct might be helpful to understand the complex responses of TREK channels since the allosteric interaction between proximal Ct and the fourth transmembrane segment is critical for the gating of TREK channels [19].

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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