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Original

Interleukin-1β suppresses activity of exogenously transfected ROMK1 K^+ channel in cultured mouse CCD cells via PKC and CaMKII pathways

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Abstract

K^+ channel ROMK1 present in the cortical collecting duct (CCD) plays an important role in renal K^+ secretion, which is known to be affected by proinflammatory cytokines. In this study, we investigated the effects of one proinflammatory cytokine, IL-1β, on activity of the exogenous ROMK1 K^+ channel in a mouse CCD cell line, M-1, using the cell-attached mode of the patch-clamp technique and Fura-2 Ca^{2+} imaging. IL-1β acutely suppressed activity of the ROMK1 K^+ channel with an increase in $[Ca^{2+}]_i$, which was blocked by IL-1-receptor antagonist and a PLC inhibitor, neomycin. Since PKC and CaMKII are known to inhibit the ROMK1 K^+ channel in a Ca^{2+}-dependent manner, we next examined effects of a PKC inhibitor, GF109203X, and a CaMKII inhibitor, KN62 on the IL-1β-induced channel suppression. Both inhibitors blocked the effect of IL-1β on channel activity. The effect of IL-1β on channel activity and $[Ca^{2+}]_i$ were observed even in the absence of bath Ca^{2+}. In addition, the depletion of $[Ca^{2+}]_i$ by thapsigargin abolished the effect of IL-1β on channel activity. In conclusion, IL-1β suppressed ROMK1 K^+ channel activity in M-1 cells via increased release of Ca^{2+} from the intracellular store and the subsequent activation of Ca^{2+}-dependent PKC and CaMKII pathways.

Key words: IL-1β, ROMK, PKC, CaMKII, calcium ion

I. Introduction

Proinflammatory cytokines are key molecules for inducing cell injury in various organs during inflammatory diseases [1]. It has been reported that proinflammatory cytokines affect the function of ion channels or transporters in renal epithelial cells [2-5]. For example, tumor necrosis factor-α (TNF-α) was shown to stimulate apical K^+ channels in rat TAL [2] and interferon-γ (IFN-γ) induced a dual phase effect, acute stimulation, and delayed suppression, on activity of an inwardly rectifying K^+ (Kir) channel in human proximal tubule cells [3]. Our previous study also revealed that interleukin-1β (IL-1β) acutely suppressed a Kir channel in cultured human proximal tubule cells [4]. In addition, exposure to several cytokines, such as IFN-γ, TNF-α, and IL-1β, for 12 hours also induced a decrease in the mRNA expression

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of K^+ channels in cortical collecting duct (CCD) cells [6]. Apical K^+ channels in principal cells of CCD are known to play a crucial role in K^+ secretion into the urine [7]. Thus, suppression of secretory K^+ channels in CCD would disturb the K^+ homeostasis in the body.

The ROMK channel cloned from rat renal outer medulla [8] was reported to be expressed in the apical membrane of distal nephrons [9]. ROMK1, one of the ROMK isoforms, is considered the major candidate for a secretory K^+ channel in CCD [7], since mice with knock-out of this isoform alone developed hyperkalemia upon high K^+ intake mice [10]). A number of studies have been carried out to clarify the mechanism by which ROMK K+ channels are regulated [8, 11-17]. Various factors such as ATP [8], PKA [11], PKC [12], CaMKII [13], some phosphatases [14], arachidonic acid [15], pH [16], and PIP2 [17] have been reported to be involved in modulating such channels' activity. However, little is known about the effect of proinflammatory cytokines on the ROMK1 K^+ channel in CCD.

In this study, we investigated the effect of IL-1β on the ROMK1 K^+ channel exogenously expressed in a transgenic mouse-derived CCD cell line, M-1, which lacks intrinsic ROMK channels [18]. We found that IL-1β suppressed the exogenous ROMK1 K^+ channel activity with an increase of $[Ca^{2+}]_i$. We further investigated the mechanism behind the IL-1β-induced suppression of channel activity, including elevation of $[Ca^{2+}]_i$.

II. Materials and methods

1. Cell culture

M-1 cells, derived from CCD of a transgenic mouse, were purchased from Summit Pharmaceutical International (Tokyo, Japan). The cells at passages 25-50 were grown in DMEM/F-12 medium (Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (Gibco) and 5 μM dexamethasone (Sigma, St. Louis, MO, USA) at 37℃ in 5% CO_2. After reaching 70%-80% confluence, the M-1 cells were dispersed with trypsin/EDTA, resuspended in the growth medium, and seeded on collagen-coated coverslips (Asahi Techno Glass, Tokyo, Japan) placed in a 35-mm-diameter dish (Falcon, Franklin Lakes, NJ, USA) for the patch-clamp experiments, or directly on a 35-mm-diameter dish with a 12-mm-diameter polymer-bottom (BMS, Tokyo, Japan) for Fura-2 Ca^{2+} imaging at a density of 1×10^5 cells/dish. After an incubation period of 24 hours, M-1 cells on the collagen-coated coverslips were transfected with human EGFP-ROMK1 cDNA constructs and incubated for another 24-48 hours. Then, the coverslip was transferred to an open bath-heating chamber mounted on an inverted microscope for the patch-clamp. M-1 cells on the polymer-bottom dish were not subjected to transfection. After an incubation period of 24-48 hours, the polymer-bottom dish was transferred to a heater platform mounted on an inverted fluorescent microscope for the Ca^{2+} imaging. All experiments were performed at 34℃.

2. Transfection of human ROMK1 gene

PCR was used to amplify cDNA representing the coding region of human ROMK1 (KCNJ1), which had been synthesized from human kidney total RNA (636529, Clontech, Mountain View, CA, USA) as a template. This coding region was subcloned into the highly efficient mammalian expression

plasmid, pCAGGS. An EGFP tag sequence was fused to the 5' -end of the coding sequence of human ROMK1. M-1 cells were transiently transfected with this expression vector, using Lipofectamine LTX and Plus reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer' s instruction.

3. Solutions and test substances

The standard bath solution contained (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 10 HEPES, with its pH adjusted to 7.35. The Ca^{2+}-free bath solution had the same ionic composition as the standard bath solution, except for $CaCl_2$ being omitted. Patch pipettes were filled with the KCl solution containing (in mM) 145 KCl, 1 $MgCl_2$, 1 EGTA, and 5 HEPES (pH 7.35). Murine IL-1β and a specific IL-1 receptor antagonist, IL-1Ra, were purchased from Peprotech EC (London, UK) and Prospec (Rehovot, Israel), respectively. A PKC inhibitor, GF109203X, and a PLC inhibitor, neomycin, were purchased from Merck (Darmstadt, Germany). A CaMKII inhibitor, KN62, and an inhibitor of endoplasmic reticulum (ER) Ca^{2+} ATPase, thapsigargin, were purchased from Wako (Osaka, Japan). A fluorescent probe for Ca^{2+}, Fura2-AM, was from Dojindo (Kumamoto, Japan). GF109203X, KN62, thapsigargin, and Fura2-AM were dissolved in DMSO as a stock solution, whereas the others were dissolved in water. Each stock solution was diluted with an appropriate amount of the bath solution before use.

4. RT-PCR

Total RNA was extracted from the untransfected M-1 cells at 70%-80% confluence, using RNeasy Mini Kit (QIAGEN, Hilden, Germany). Then, the RNA sample (1 μg) was reverse-transcribed, using the RNA PCR kit AMV Ver.3.0 (TaKaRa Bio, Otsu, Japan). The resultant cDNA (1 μl) and mouse kidney cDNA(1 μl: GenoStaff, Tokyo, Japan) were amplified with AmpliTaq GOLD 360 Master Mix (Applied Biosystems, Foster City, CA, USA) and specific primers (0.4 μM). The primers for mouse type 1 IL-1 receptor (IL-1R1) were 5 ' -ACCCCCATATCAGCGGACCG-3 ' (forward) and 5 ' -TTGCTTCCCCCGGAACGTAT-3 ' (reverse), amplifying a 429-bp product. These sequences were designed by referring to a previous study [19]. The primers for mouse ROMK were 5' -GATCTCCCAGAGTTCTAC-3 ' (forward) and 5 '-AATAACTGGTGTTGTCG GGA -3' (reverse), amplifying a 510-bp product. The primers for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5 ' -ACCACAGTCCATGCCATCAC-3 ' (forward) and 5 ' -TCCACCACCCTGTTGCTGCA-3 ' (reverse), amplifying a 450-bp product. PCR was carried out using the GeneAmp PCR system (Applied Biosystems). PCR conditions for mouse type 1 IL-1 receptor were 95 ℃ for 5 min, 32 cycles of 95 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 30 s, and finally 72 ℃ for 7 min. PCR conditions for mouse ROMK were 95 ℃ for 5 min, followed by 36 cycles of 95℃ for 30 s, 60℃ for 30 s: and 72℃ for 30 s, and finally 72℃ for 7 min. In each PCR experiment, we also included a sample without reverse transcription in parallel, which served as a negative control. PCR products were electrophoresed on a 2% agarose gel containing 0.1 μg/ml ethidium bromide and visualized with ultraviolet (UV) light with a wavelength of 312 nm.

5. Patch-clamp technique

Channel recordings were performed in cell-attached patches applied to the surface membrane of single M-1 cells, with the pipette-holding potential set at 0 mV. Patch pipettes were made of borosilicate glass capillaries (GC150-7.5: Warner, Hamden, CT, USA) on a two-step puller (model PP830: Narishige, Tokyo, Japan), and filled with the KCl solution. The pipette resistance ranged from 3 to 5 MΩ. Current signals were measured with a patch-clamp amplifier, Axopatch 200B (Molecular Devices, Sunnyvale, CA, USA), and recorded on a DAT (RD-120TE: TEAC, Tokyo, Japan). Current signals were then low-pass-filtered at 500 Hz by the 3611 Multifunction Filter (NF electronic instruments, Tokyo, Japan) and digitized at 2.5 kHz through an interface (Digidata 1322A: Molecular Devices, Sunnyvale, CA, USA). The acquired data were analyzed with the pCLAMP8 software (Molecular Devices). Channel activity was determined by NPo, which was calculated from an amplitude histogram as:

$$NP_o = \sum_{n=1}^{N} n \cdot t_n$$

where N is the maximum number of open channels observed in the patch, Po is the open probability, n is the number of channels observed at the same time, and tn is the probability that n channels are simultaneously open. We calculated normalized channel activity (NPo,e/NPo,c) for convenience in comparing the channel activity in experimental conditions with that in controls. NPo,c and NPo,e are the channel activities under control and experimental conditions, respectively. NPo,c was determined from a 10-20-s sampling period just before adding the substance when the steady state lasted for at least 60 s. NPo,e was determined from a 10-20 s sampling period extracted from the steady state for at least 20-30-s made by the experimental substance.

6. Intracellular Ca^{2+} measurement

The intracellular Ca^{2+} concentration was estimated with Fura2-AM, using the InCyt Basic IM imaging system (Intracellular Imaging, Cincinnati, OH, USA). M-1 cells on the polymer-bottom dishes were loaded with 4 μM Fura-2AM in the growth medium for 20 min at 37℃. Then, the dishes were washed with the standard bath solution and placed on a heater platform mounted on a fluorescent microscope. The Fura-2-loaded cells were excited by alternating exposure to 340-nm and 380-nm UV light, and the fluorescent emission at 510 nm (F340 / F380) for estimating $[Ca^{2+}]_i$ was recorded every 5 s by a CCD camera, with the objective set at × 40. The control value was obtained from the mean value of a 30 s of data before application of the substance.

7. Statistical analysis

Data are expressed as mean ± SEM. Student's t-test or ANOVA in conjunction with a Bonferroni t-test was used for statistical comparisons. A p value of less than 0.05 was considered significant.

III. Results

1. Expression of IL-1R1 mRNA

We first investigated IL-1R1 mRNA expression in M-1 CCD cells using reverse RT-PCR analysis. IL-1R1 has been reported to be expressed widely and heterogeneously in the kidney [20], e.g. in renal tubules and

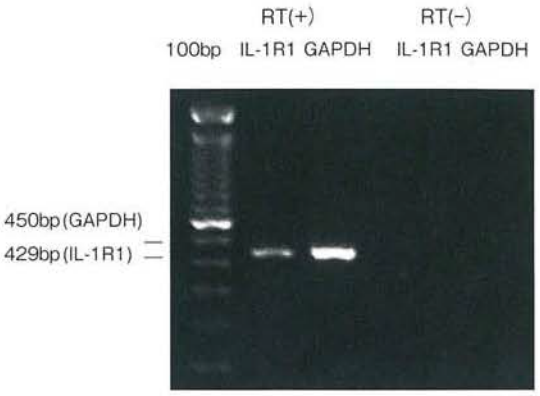

Fig. 1. Expression of IL-1R1 mRNA in cultured M-1 CCD cells. RT (+) and RT (-) represent samples with and without reverse transcription, respectively.

glomeruli [21]. As shown in Fig. 1, mRNA expression of IL-1R1 in cultured M-1 cells was also confirmed.

2. Detection of transfected ROMK1 K^+ channel in M-1 cells

As previously reported, the ROMK-like K^+ channel has not been observed in M-1 cells [18]. We also confirmed that ROMK mRNA was not expressed in M-1 cells, as indicated in Fig. 2A, whereas it was expressed in products from mouse kidney CCD, as determined by RT-PCR analysis. We transfected the human ROMK1 gene tagged with EGFP into M-1 cells. We observed the expression of ROMK1 K^+ channels with EGFP in single M-1 cells 24-48 hours after transfection. Examples of a cell imaged using bright-field microscopy and fluorescent microscopy are shown in Figs. 2B and 2C, respectively. We applied a patch pipette to an M-1 cell exhibiting fluorescence: the K^+ channel current observed in this cell is shown in Fig. 2D. The I-V relationship of the observed channel was examined in cell-attached patches. Fig. 2E presents an example of traces obtained from a recording in five steps of different Vp. Data of the traces with different Vp (n = 4) were pooled and the slope conductance from each trace was calculated. The inward conductance of the observed K^+ channel from -30 mV to 30 mV was 42 ± 4 pS and the outward conductance from -120 mV to -90 mV was 11 ± 2 pS. This inward conductance and the rectification were almost identical to those of the ROMK1 K^+ channel [8]. Furthermore, P_o of the inward current was not altered, suggesting that the channel activity was not voltage-dependent, although outward channel current was markedly depressed and NP_o seems to be slightly reduced.

3. Effect of IL-1 β on ROMK1 K^+ channel

Channel currents were recorded by cell-attached patches applied to the exogenously transfected ROMK1 K^+ channel, which was expressed on the surface membrane of single M-1 principal cells. IL-1β added to the bath at 5 ng/ml suppressed channel activity within a few minutes, and it was recovered to the control level after wash-out as shown in Fig. 3A. Figure 3D shows the summarized data, indicating that IL-1β significantly decreased the probability of the ROMK1 K^+ channel being open to 27% ± 8% (n=13) compared with that of the control. To confirm that the channel suppression induced by IL-1β was receptor-mediated, we examined the effect of IL-1Ra. Figure 3B shows that the administration of IL-1β (5 ng/ml) in the presence of IL-1Ra (500 ng/ml) completely prevented the suppressive effect of IL-1β. IL-1Ra alone did not affect channel activity. A summary of the data is shown in Fig. 3E. IL-1Ra and IL-1β + IL-1Ra did not cause any differences compared to the control. IL-1β -induced channel suppression was restored following the addition of IL-1Ra, as shown

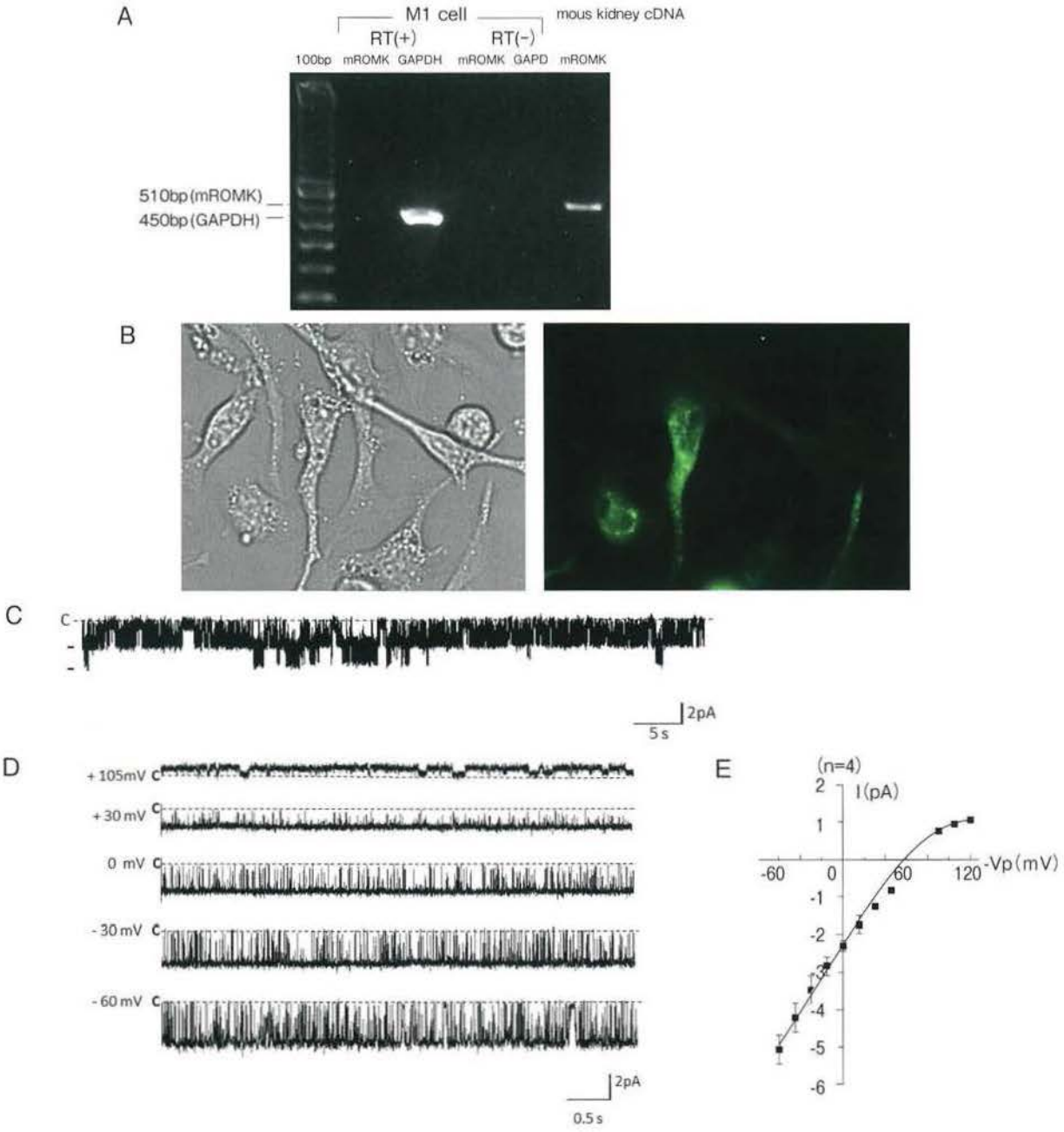

Fig. 2.

A: RT-PCR analysis of mouse ROMK (mROMK) mRNA in cultured M-1 CCD cells. RT (+) and RT (-) represent samples with and without reverse transcription, respectively. Mouse kidney cDNA was employed to validate primers for mROMK.

B: Exogenously transfected human ROMK1 K^+ channels in single M-1 cells. Bright-field image of M-1 cells (× 400) is on the left and the same image of cells from fluorescent microscopy (× 400) is on the right.

C: Current recording obtained from cell-attached patch of the cell indicated by the arrow in the right image in Fig. B. Current levels of the channel are indicated by short bars (left), and closed channel state is indicated by C (top left).

D: Channel recordings of the exogenously transfected human ROMK1 K^+ channel in M-1 cells obtained from a cell-attached patch at several different Vp.

E: The I-V relationship of the channel was obtained from four cell-attached patches under the conditions with the standard bath solution.

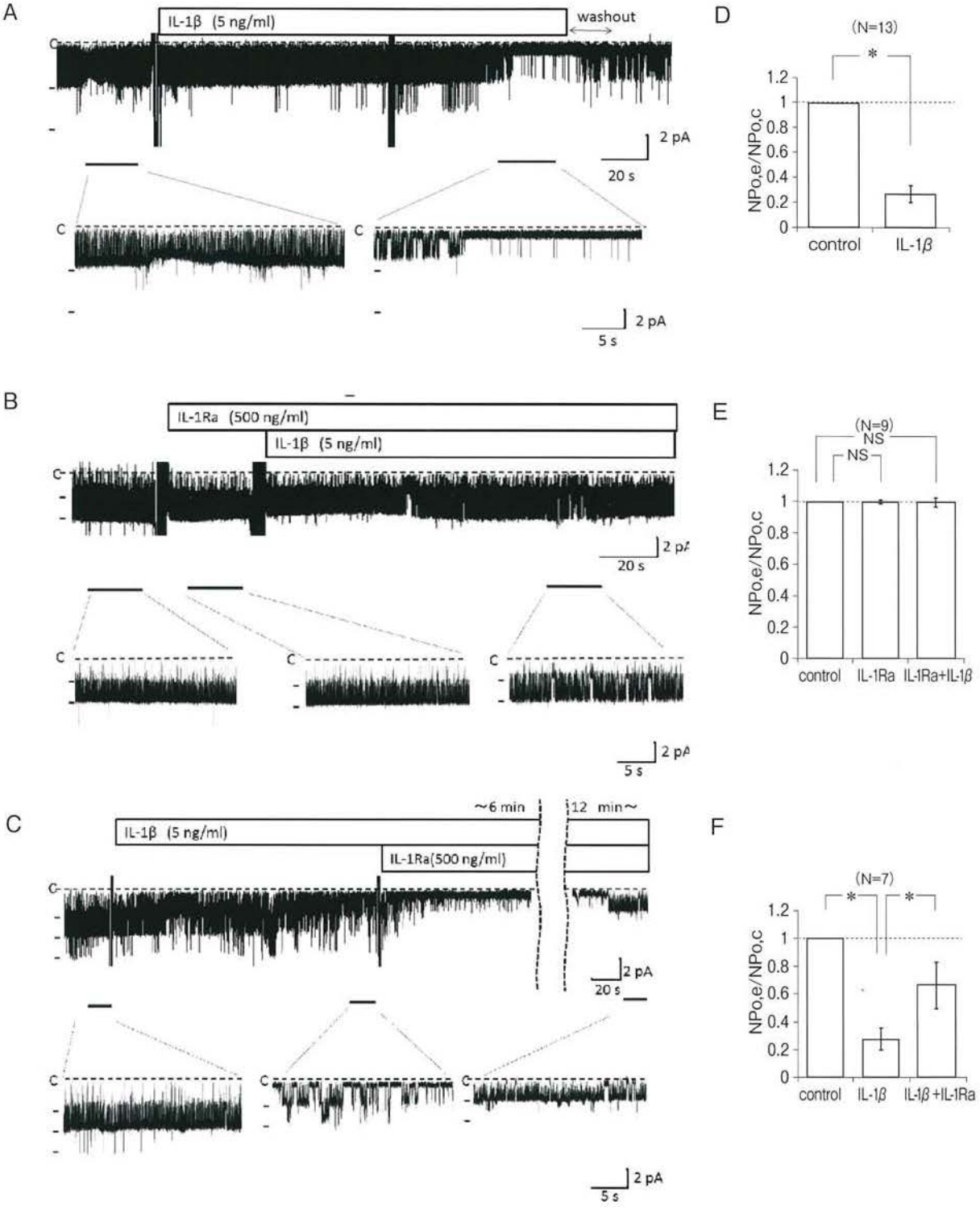

Fig. 3. Effect of IL-1β on ROMK1 K^+ channel activity.

A. Suppression of the channel activity by IL-1β .

B. Blockade of the suppressive effect of IL-1β by IL-1Ra.

C. Restoration of channel activity by IL-1Ra in the presence of IL-1β .

D–F. Summarized data obtained from experiments similar to those shown in A–C, respectively.

*: Significantly different ($p<0.05$) compared with the control.

NS: not significant.

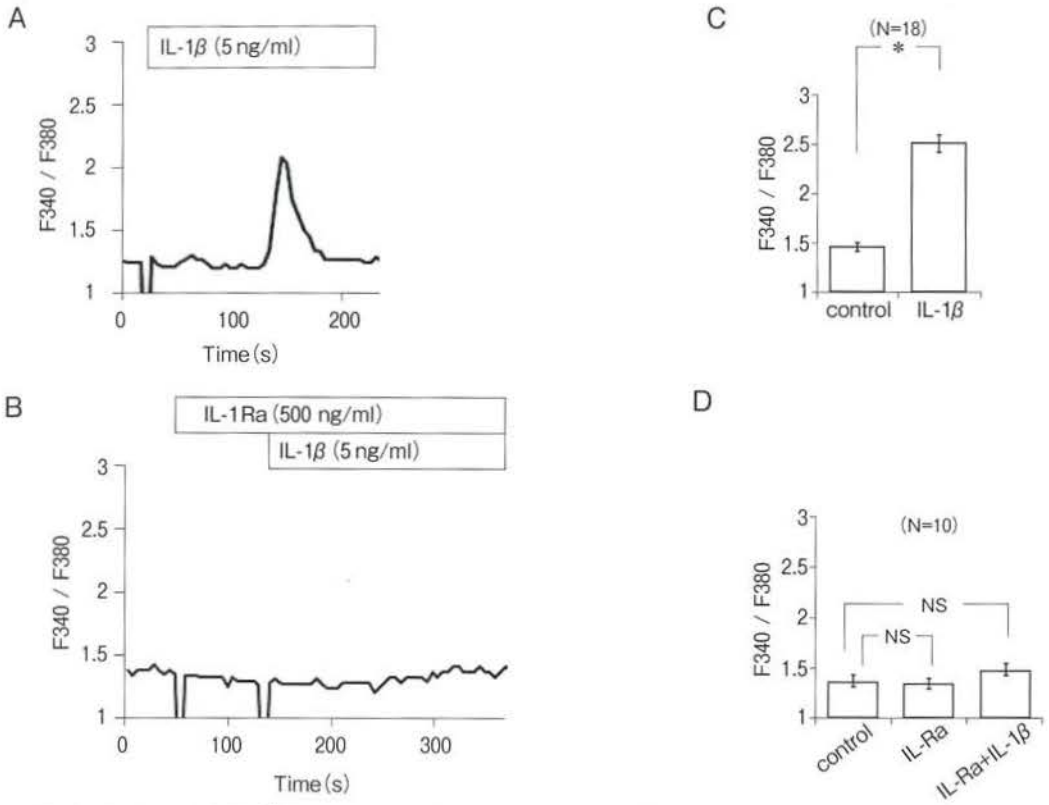

Fig. 4. Changes in $[Ca^{2+}]_i$ in response to IL-1 β in M-1 cells.
A. Effect of IL-1 β on $[Ca^{2+}]_i$.
B. Effect of IL-1 β on $[Ca^{2+}]_i$ in the presence of IL-1Ra.
C and D. Summarized data obtained from experiments similar to those shown in A and C respectively.
*: Significantly different ($p<0.05$) compared with the control.
NS: not significant.

in Fig. 3C. Data of the channel suppression by IL-1β and its restoration by IL-1Ra are summarized in Fig. 3F. These results suggest that the IL-1β -induced acute suppression of the ROMK1 K^+ channel was dependent on specific receptor-mediated processes.

4. Effect of IL-1 β on $[Ca^{2+}]_i$ in M-1 cells

It has been reported that IL-1β increases $[Ca^{2+}]_i$ in various cells, including human proximal tubule cells [4, 22, 23]. Therefore, we also examined the changes in $[Ca^{2+}]_i$ in response to IL-1β in M-1 cells using Fura-2AM Ca^{2+} imaging. As shown in Fig. 4A, a transient increase in $[Ca^{2+}]_i$ was observed in a few minutes after the application of IL-1β in the standard bath. The peak values of $[Ca^{2+}]_i$ in response to IL-1β were significantly higher than those of the control. Fig. 4B shows that the application of IL-1β in the presence of the IL-1Ra did not change $[Ca^{2+}]_i$.

5. Effects of inhibitors of PKC and CaMKII on the IL-1β -induced channel suppression and $[Ca^{2+}]_i$

The above data suggest that an increase in $[Ca^{2+}]_i$ induced by IL-1β would cause channel suppression. Since it is known that increased $[Ca^{2+}]_i$ would stimulate PKC- and CaMKII-dependent phosphorylation and inhibit the activity of ROMK-like K^+ channels [13, 24], we examined the effects of IL-1β on the ROMK1

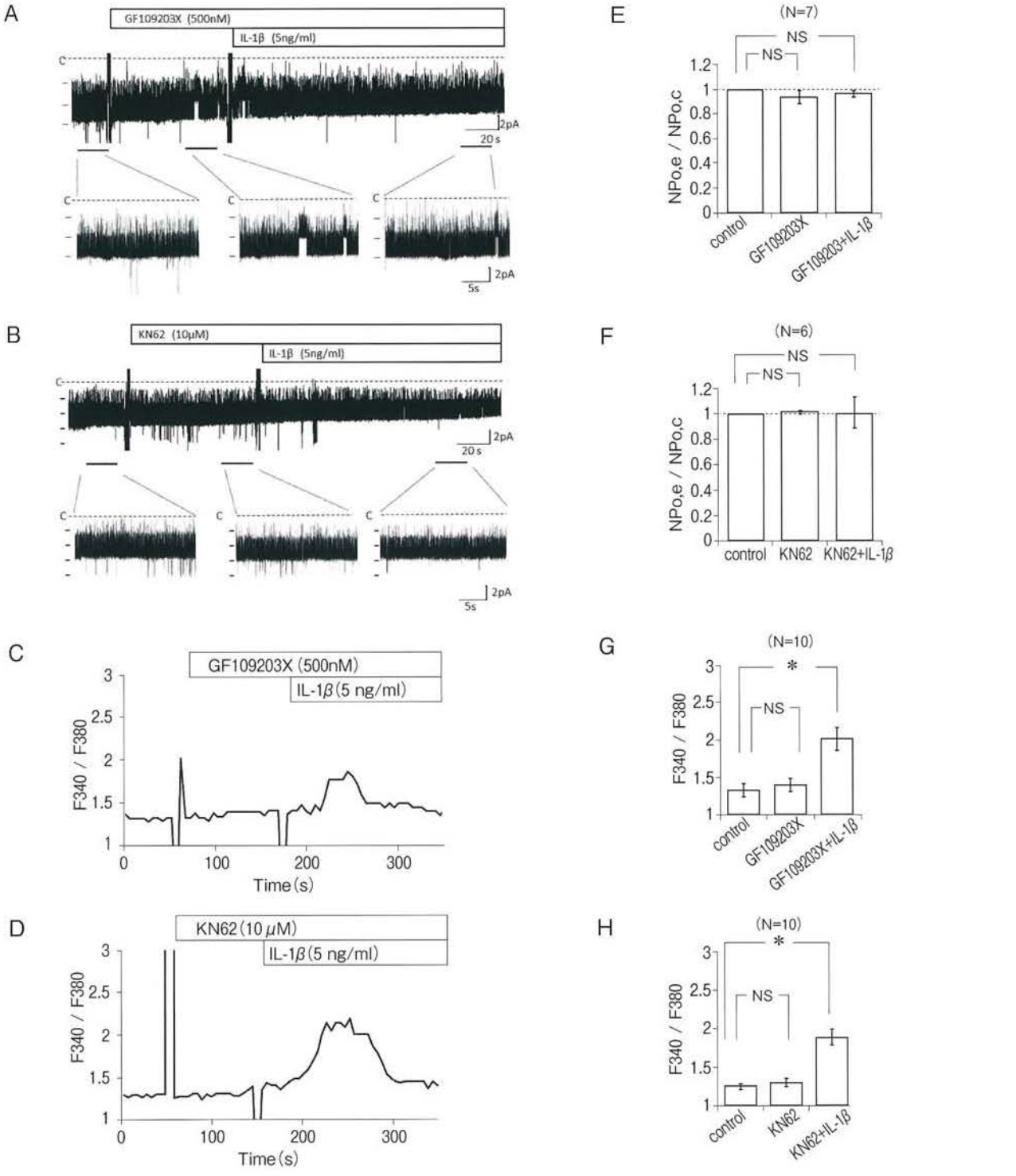

Fig. 5. Effect of a PKC inhibitor and a CaMKII inhibitor on the IL-1β -induced ROMK1 K^+ channel suppression and on the IL-1β -induced rise in $[Ca^{2+}]_i$ in M-1 cells.

A and B: Effect of IL-1β in the presence of a PKC inhibitor, GF109203X, and a CaMKII inhibitor, KN62, respectively.

C and D: Changes in $[Ca^{2+}]_i$ in response to IL-1β in the presence of GF109203X and KN62, respectively.

E–H: Summarized data obtained from experiments similar to those shown in A–D, respectively.

*: Significantly different ($p<0.05$) compared with the control.

NS: not significant.

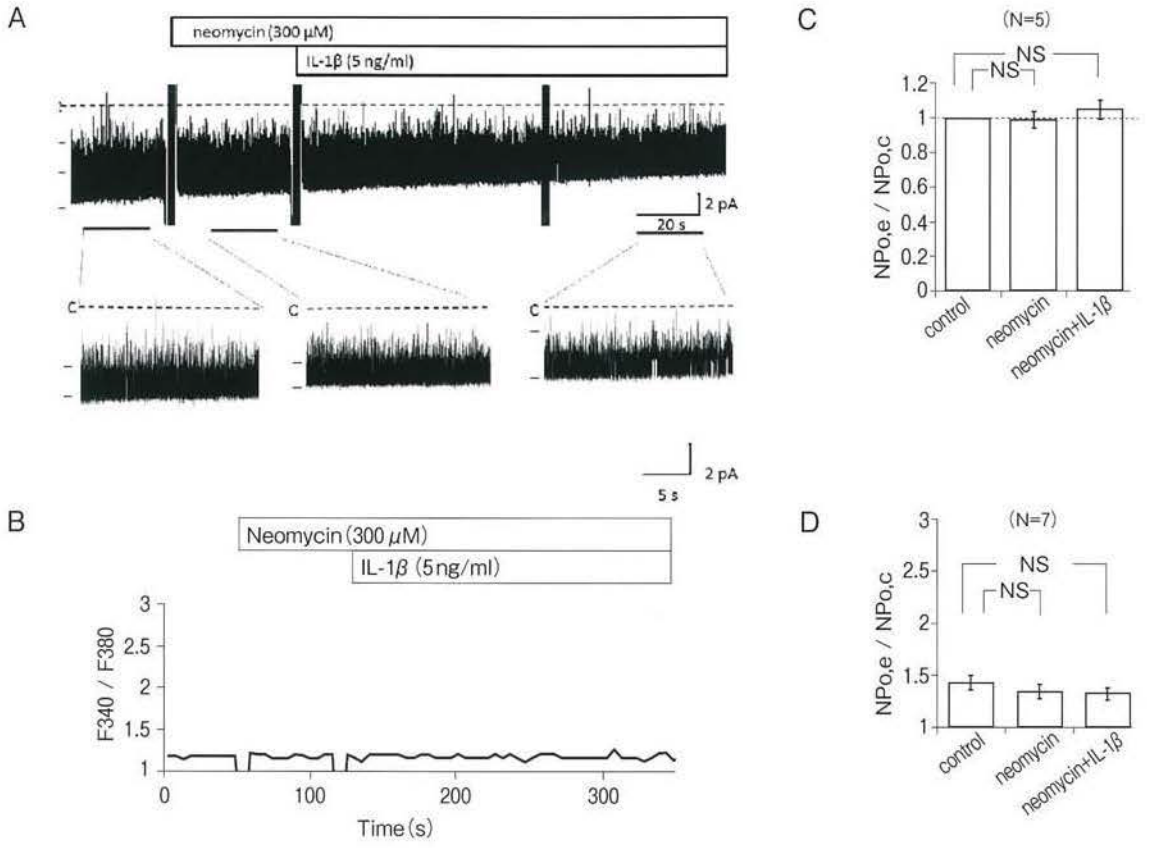

Fig. 6. Blockade of IL-1β -induced change in channel activity and $[Ca^{2+}]_i$ by a PLC inhibitor, neomycin.
A: Effect of IL-1β on channel activity in the presence of neomycin.
B: Changes in $[Ca^{2+}]_i$ in response to IL-1β in the presence of neomycin.
C and D: Summarized data obtained from experiments similar to those shown in A and B, respectively.
*: Significantly different ($p<0.05$) compared with the control.
NS: not significant.

K^+ channel activity and $[Ca^{2+}]_i$ in the presence of respective inhibitors. We used GF109203X as a PKC inhibitor, and KN62, as a CaMKII inhibitor. Figs. 5A and 5B show representative current recordings in response to GF109203X (500 nM) and KN62 (10 μM), respectively. Pretreatment of either GF109203X or KN62 did not affect channel activity and these two substances both abolished the IL-1β -induced channel suppression. Data on the effects of GF109203X and KN62 on IL-1β -induced channel suppression are summarized in Figs. 5E and 5F, respectively. Figs. 5C and 5D present the effects of IL-1β in the presence of GF109203X and KN62 on $[Ca^{2+}]_i$ in M-1 cells, respectively. Neither inhibitor affected the IL-1β -induced transient increase in $[Ca^{2+}]_i$. Data of Figs. 5C and 5D are summarized in Figs. 5G and 5H, respectively. These results suggest that IL-1β -induced channel suppression is not be directly mediated by an increase in $[Ca^{2+}]_i$ but by the activation of PKC and CaMKII.

6. Involvement of PLC in IL-1β -induced suppression and a rise in $[Ca^{2+}]_i$

PLC activates PKC in two ways, hydrolyzing PIP2 into IP3 and DAG, the former activating PKC indirectly by a rise

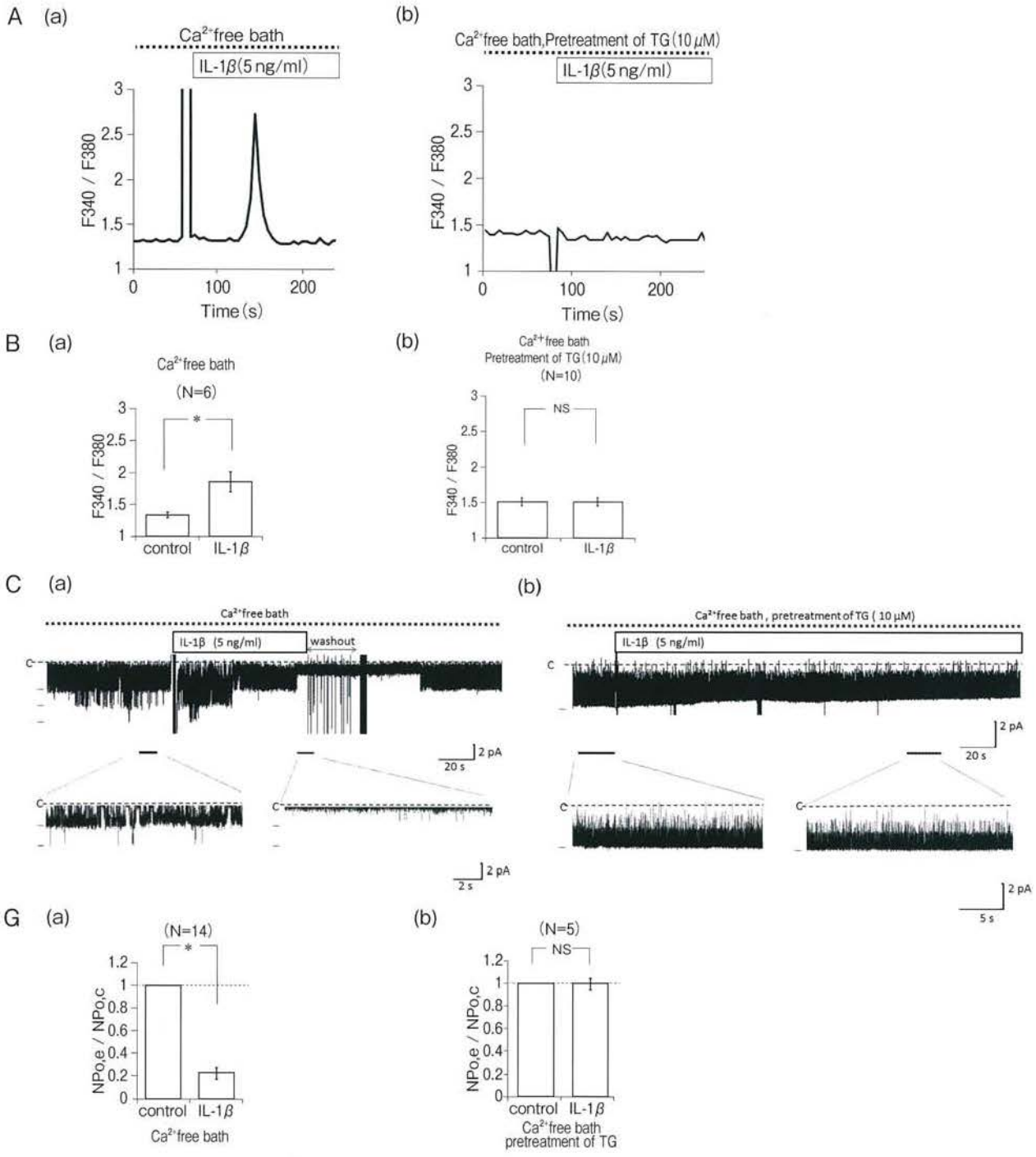

Fig. 7. Effect of IL-1 β on $[Ca^{2+}]_i$ and channel activity with or without pretreatment of thapsigargin (TG) in nominally Ca^{2+}-free bath solution.

A: Changes in $[Ca^{2+}]_i$ in response to IL-1β in M-1 cells without TG (a) and after 10 min of pretreatment with TG (b) in Ca^{2+}-free bath.

C: Effect of IL-1β on ROMK1 K^+ channel in Ca^{2+}-free bath without TG (a) and after 10 min pretreatment with TG (b) in Ca^{2+}-free bath.

B and D: Summarized data of A(a), A(b) , C(a), and C(b) obtained from the similar experiments are shown in B(a), B(b), D(a), and D(b), respectively.

*: Significantly different ($p<0.05$) compared with the control.

NS: not significant.

in $[Ca^{2+}]_i$ and the latter activating PKC directly [25]. It has been reported that the PLC inhibitor prevented the IL-1β -induced suppression of a Kir channel in cultured proximal tubule cells [4]. Thus, we also examined the effect of a PLC inhibitor, neomycin, on the IL-1β -induced suppression of the ROMK1 K^+ channel activity. Fig. 6A shows that IL-1β had no effect on channel activity in the presence of neomycin (300 μM). The data are summarized in Fig. 6C, suggesting that IL-1β -induced channel suppression was mediated by PLC-dependent processes. Furthermore, we investigated whether the IL-1β -induced rise in $[Ca^{2+}]_i$ is mediated by the PLC pathway using neomycin. Fig. 6B shows that neomycin also completely blocked the rise in $[Ca^{2+}]_i$, revealing that the IL-1β -induced rise in $[Ca^{2+}]_i$ is also mediated by the PLC pathway.

7. Involvement of extracellular and intracellular Ca^{2+} in the IL-1β -induced rise in $[Ca^{2+}]_i$

First, we examined changes in $[Ca^{2+}]_i$ in response to IL-1β in the absence of extracellular Ca^{2+}. As shown in Fig. 7A(a), IL-1β rose $[Ca^{2+}]_i$ even in the absence of extracellular Ca^{2+}, suggesting that this phenomenon was not dependent on Ca^{2+} influx from the bath. Next, we examined the involvement of the intracellular Ca^{2+} store in the IL-1β -induced rise in $[Ca^{2+}]_i$. It is known that a Ca^{2+}-ATPase inhibitor, thapsigargin, depletes Ca^{2+} of the store in the cell [26]. After incubating M-1 cells with thapsigargin (10 μM) for 10 min in a Ca^{2+}-free bath, the application of IL-1β did not change $[Ca^{2+}]_i$ as shown in Fig. 7A(b). These findings are summarized in Fig. 7B, suggesting that the IL-1β -induced increase in $[Ca^{2+}]_i$ was derived from the intracellular Ca^{2+} store. Since IL-1R1-mediated release of stored Ca^{2+} depends on IP3, these findings suggest that the IL-1β -induced rise in $[Ca^{2+}]_i$ is mediated by PLC/IP3 signaling [27]. In addition, pretreatment with thapsigargin for 10 min abolished IL-1β -induced channel suppression, while IL-1β suppressed channel activity without pretreatment with thapsigargin even in the absence of bath Ca^{2+} (Fig. 7C). Therefore, we conclude that the IL-1β -induced rise in $[Ca^{2+}]_i$ is derived from stored Ca^{2+}, which activates PKC and CaMKII, resulting in channel suppression.

IV. Discussion

The effects of proinflammatory cytokines on renal ion channels or transporters have been investigated [2, 5, 28, 29]. A recent study reported that IL-1β , TNF-α , and IFN-γ decreased the gene expression of several transporters in the renal cortex, including the ROMK K+ channel, in cultured CCD cells [6]. It has also been reported that IL-1β acutely suppressed K^+ channel activity in human proximal tubule cells [4]. Since the ROMK channel is responsible for K^+ secretion in CCD [7], not only IL-1β -induced decrease in the expression of ROMK but also the acute effect of IL-1β on channel activity of ROMK might disrupt K^+ homeostasis. However, little is known about the cytokine-induced acute effect on K^+ channels in CCD. In this study, we thus focused on the acute effect of IL-1β on ROMK1 K^+ channel activity in cultured M-1 cells.

The confluent layered cultured M-1 cell system is a useful tool for investigating

CCD functions, including the modulation of ion channels or transporters [30-32]. However, the M-1 cells that we used were single M-1 cells, but not polarized confluent M-1 cells. Moreover, since ROMK mRNA was not expressed in cultured M-1 cells, as shown in Fig. 2A, we transfected the human ROMK1 K^+ channel into M-1 cells. These experimental conditions might not be appropriate for investigating the functional effects of IL-1 β on CCD. Although our data did not show the function of CCD, experiments using single M-1 cells should reveal the mechanism behind IL-1 β -induced changes in ROMK K+ channel activity, at least at the cellular level.

1. Effects of IL-1 β on ROMK 1 K^+ channel

In the present study, we demonstrated that IL-1β inhibits the activity of the transfected ROMK1 K^+ channel in cultured M-1 cells via the activation of PKC and CaMKII. It is well known that IL-1β binds to its specific receptor, IL-1R1, of target cells, initiating various signal transduction pathways [33]. IL-1Ra, which belongs to the IL-1 superfamily, also binds to IL-1R1. IL-1Ra is not a trigger for various signals, but a competitive ligand of IL-1β [34]. It has been reported that the mRNA expression of the ROMK channel in cultured CCD cells was suppressed 12 hours after the administration of several cytokines including IL-1β [6]. In our study, the application of IL-1 β gradually decreased channel activity in a few minutes, as shown in Figs. 3A and 3C. IL-1β -induced channel suppression was reactivated by the wash-out of IL-1β or the application of a high concentration of IL-1Ra. These findings indicate that the application of IL-1β for a few minutes directly inhibits ROMK K^+ channel activity via receptor-mediated processes. Taking a previous study [6] into account, it is likely that the exposure of M-1 cells to IL-1β would suppress ROMK1 K^+ channel function not only via the downregulation of mRNA expression but also via the acute attenuation of channel activity.

The affinity of the receptor, IL-1R1, to IL-1Ra is over 30 times greater than that to IL-1β [35]. Thus, the administration of a high concentration of IL-1Ra would be sufficient to prevent IL-1β signaling. It might depend on the relative concentrations between IL-1β and IL-1Ra whether acute signaling of IL-1β would affect cellular functions.

2. Modulation of ROMK1 K^+ channel activity via PKC and CaMKII

We demonstrated in this study that IL-1β suppressed ROMK1 K^+ channel activity with an increase in $[Ca^{2+}]_i$. IL-1β -induced channel suppression was abolished by a PKC inhibitor and a CaMKII inhibitor. Depletion of $[Ca^{2+}]_i$ also abolished IL-1 β -induced channel suppression. Thus, suppression of the channel activity was induced by PKC and CaMKII, which were stimulated by the rise in $[Ca^{2+}]_i$. PKC-mediated acute inhibition of K^+ channels in the kidney has been reported, such as in native ROMK-like K^+ channels in the apical membrane of rat principal cells of CCD [24], a Kir channel in cultured human proximal tubule cells [4], and a 90 pS K^+ channel in cultured opossum kidney proximal tubule cells [36]. ROMK1 K^+ channel is also known to be inhibited by a PKC-mediated pathway [12]. As well as PKC, CaMKII is activated by a rise in $[Ca^{2+}]_i$. As for the modulation of K^+ channels, it has been reported that CaMKII is also involved in suppressing ROMK-like K^+

channel activity in rat CCD principal cells [13]. In this previous study, it was demonstrated that Ca^{2+}-induced K^+ channel suppression was mediated by both CaMKII and Ca^{2+}-dependent PKC [13].

It has been demonstrated that Ca^{2+}-dependent suppression of K^+ channel activity was not completely blocked by a PKC inhibitor or a CaMKII inhibitor [13, 36, 37] but was completely blocked by the application of both PKC and CaMKII inhibitors [13]. However, in our study, either PKC inhibitor or CaMKII inhibitor both completely blocked the suppressive effect of IL-1 β on ROMK1 K+ channels, which is inconsistent with previous studies [13, 36, 37]. However, recent studies showed a plausible interaction between PKC and CaMKII [38, 39]. That is, PKC indirectly stimulates CaMKII phosphorylation [38], suggesting that the inhibition of PKC might inhibit the production of phospho-CaMKII. Namely, it is likely that PKC inhibitor could inhibit CaMKII activation, resulting in the complete suppression of IL-1 β -induced channel suppression by PKC inhibitor alone. On the other hand, KN62, known as a CaMKII-specific inhibitor, has been reported to inhibit PKC- mediated phosphorylation of GluA1 in hippocampal neurons [39]. This previous study [39] suggests that CaMKII inhibitor KN62 could inhibit not only CaMKII but also PKC, which would result in complete inhibition of IL-1 β -induced channel suppression by KN62. Although involvement of the interaction between PKC and CaMKII or the inhibitory effect of KN62 on PKC in M-1 cells is still unknown, our data certainly show that both PKC inhibitor alone and CaMKII inhibitor alone completely inhibit IL-1 β -induced channel suppression. Further experiments will be necessary to clarify the interaction of PKC and CaMKII in M-1 cells.

3. ROMK1 K^+ channel suppression via PLC pathway and $[Ca^{2+}]_i$ elevation

After the binding of IL-1 β to IL-1R1, the recruitment of IL-1 receptor accessory protein (IL-1RAcP) leads to the formation of a trimeric complex 33). This complex initiates several downstream pathways including PLC, IL-1 receptor-associated kinase (IRAK), and phosphoinositide 3-kinase (PI3K) [33]. Among them, the IRAK pathway would be excluded since IRAK is not related to the acute signaling pathways, but to the transcription and expression signals [33]. As for the effect of the PI3K pathway on ROMK1 K^+ channel activity, it has been reported that inhibition of PI3K stimulates ROMK channel activity in CCD [40]. Thus, PI3K may be involved in IL-1β -induced ROMK1 K^+ channel suppression. However, PI3K is related to tyrosine kinase stimulation [40], but not to the stimulation of PKC or CaMKII. As mentioned above, since PKC inhibitor and CaMKII inhibitor abolished IL-1β -induced channel suppression, PI3K would not have participated in the IL-1β -induced ROMK1 K^+ channel suppression in this study. Therefore, we focused on the PLC pathway because our previous study showed the involvement of the PLC pathway in the rapid inhibitory effect of IL-1β -induced channel suppression on a renal Kir channel [4].

In this study, the PLC inhibitor, neomycin, prevented IL-1β -induced channel suppression and also blocked the rise in $[Ca^{2+}]_i$. There are three downstream pathways following PLC activation [22, 41]. First, DAG induces a rise in $[Ca^{2+}]_i$ via the TRPC channel [41],

resulting in Ca^{2+} inflow and activating PKC and CaMKII. Second, IP3, generated from PIP2, induces a rise in $[Ca^{2+}]_i$ from the store, resulting in the activation of PKC and CaMKII. Third, DAG, also generated from PIP2, directly activates PKC [22]. In a Fura-2 Ca^{2+} imaging experiment, an IL-1β -induced $[Ca^{2+}]_i$ increase in M-1 cells was observed in the absence of Ca^{2+} in bath solution. This suggests that extracellular Ca^{2+} is not necessary for IL-1β -induced $[Ca^{2+}]_i$elevation in M-1 cells. Thus, the TRPC channel pathway would not play a crucial role in $[Ca^{2+}]_i$ caused by IL-1β , even though TRPC channels are expressed in cultured M-1 cells [42] or other CCD cells [43]. In our other experiment using thapsigargin, IL-1β had no effect on $[Ca^{2+}]_i$ in M-1 cells, suggesting that the IL-1β -induced rise in $[Ca^{2+}]_i$ is mainly derived from stored Ca^{2+}. A number of previous studies reported that IL-1β induced Ca^{2+} release from IP3R, such as in mouse astrocytes [22], in mouse hippocampus [23], and in human proximal tubule cells [4]. Therefore, we conclude that IL-1β induces an increase in $[Ca^{2+}]_i$ via the PLC/IP3 pathway. This is supported by the previous report describing how IL-1β -activated PLC signaling leads to the accumulation of IP3 in human airway smooth muscle cells [44]. Finally, we investigated the effect of IL-1β on ROMK1 K^+ channel activity in the absence of bath Ca^{2+} with or without the depletion of stored Ca^{2+} by thapsigargin. IL-1β suppressed ROMK1 K^+ channel activity in Ca^{2+}-free bath without thapsigargin pretreatment, but did not do so after such pretreatment, which rules out the involvement of the DAG direct pathway. These findings strongly suggest that a rise in $[Ca^{2+}]_i$ is essential for IL-1β -induced ROMK1 K^+ channel suppression. Moreover, we also confirmed that both Ca^{2+}-dependent PKC and CaMKII are involved in IL-1β -induced ROMK1 K^+ channel suppression. Taking these findings together, IL-1β acutely suppressed the activity of transfected ROMK1 K^+ channels in M-1 cells via an increase in $[Ca^{2+}]_i$ from the store and Ca^{2+}-dependent PKC/CaMKII-mediated pathways.

V. Conclusion

In this study, we clarified the signaling pathway transduction induced by IL-1β in cultured CCD cells. IL-1β binds to the membrane receptor, IL-R1, and induces an increase in $[Ca^{2+}]_i$ from the store. Subsequent activation of Ca^{2+}-dependent PKC and CaMKII is involved in the acute suppression of exogenously transfected ROMK1 K^+ channels.

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インターロイキン－1βは培養マウス集合管細胞に遺伝子導入したヒト ROMK1 K^+ チャネル活性を PKC 及び CaMKII を介する経路で抑制する

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要旨

ROMK1 K^+ チャネルは腎集合管主細胞に存在する主な K^+ 分泌路であり，様々な炎症性サイトカインの影響を受けることが知られている．本研究では代表的な炎症性サイトカインである IL-1β が，マウス腎集合管細胞株（M-1 細胞）へ遺伝子導入した ROMK1 K^+ チャネルの活性に及ぼす影響とその機序についてパッチクランプ法と蛍光 Ca^{2+} イメージング法を用いて検討した．細胞外 Ca^{2+} の有無に関わらず IL-1β 投与によりチャネル活性低下と $[Ca^{2+}]_i$ の一過性上昇を認め，受容体拮抗薬である IL-1Ra，PLC 阻害剤である neomycin 前投与，あるいは細胞外 Ca^{2+}-free 条件下における thapsigargin 前処置による Ca^{2+} ストア枯渇で抑制された．ROMK1 K^+ チャネル活性の Ca^{2+} 依存性抑制因子として知られている PKC と CaMKII の各々の阻害剤である GF109203X と KN62 を前投与したところ，IL-1β によるチャネル活性抑制は阻害され，$[Ca^{2+}]_i$ 上昇がみられた．以上より IL-1β による ROMK1 K^+ チャネルの活性低下には IP3 を介したストア由来の $[Ca^{2+}]_i$ 上昇と，それに続く PKC / CaMKII 活性化が関与することが明らかとなった．