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Role of Calcineurin-Mediated Dephosphorylation in Modulation of an Inwardly Rectifying K^+ Channel in Human Proximal Tubule Cells

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Abstract Activity of an inwardly rectifying K^+ channel with inward conductance of about 40 pS in cultured human renal proximal tubule epithelial cells (RPTECs) is regulated at least in part by protein phosphorylation and dephosphorylation. In this study, we examined involvement of calcineurin (CaN), a Ca^{2+}/calmodulin (CaM)–dependent phosphatase, in modulating K^+ channel activity. In cell-attached mode of the patch-clamp technique, application of a CaN inhibitor, cyclosporin A (CsA, 5 μM) or FK520 (5 μM), significantly suppressed channel activity. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) estimated by fura-2 imaging was elevated by these inhibitors. Since inhibition of CaN attenuates some dephosphorylation with increase in $[Ca^{2+}]_i$, we speculated that inhibiting CaN enhances Ca^{2+}-dependent phosphorylation, which might result in channel suppression. To verify this hypothesis, we examined effects of inhibitors of PKC and Ca^{2+}/CaM-dependent protein kinase-II (CaMKII) on CsA-induced channel suppression. Although the PKC inhibitor GF109203X (500 nM) did not influence the CsA-induced channel suppression, the CaMKII inhibitor KN62 (20 μM) prevented channel suppression, suggesting that the channel suppression resulted from CaMKII-dependent processes. Indeed, Western blot analysis showed that CsA increased phospho-CaMKII (Thr286), an activated CaMKII in inside–out patches, application of CaM (0.6 μM) and CaMKII (0.15 U/ml) to the bath at 10^{-6} M Ca^{2+} significantly suppressed channel activity, which was reactivated by subsequent application of CaN (800 U/ml). These results suggest that CaN plays an important role in supporting K^+ channel activity in RPTECs by preventing CaMKII-dependent phosphorylation.

Keywords Potassium channel · Protein phosphatase · Cyclosporin A · CaMKII · Kidney

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Introduction

The physiological significance and regulatory mechanisms of ion channels including many types of K^+ channels have been investigated in a variety of cell membranes. In renal tubular epithelia, several kinds of K^+ channels are reported in both apical and basolateral membranes along the nephron segments (Wang et al. 1997), and the physiological importance and molecular characteristics of these channels have been investigated (Hebert et al. 2005). Among several segments of the nephron, the proximal tubules predominantly expressed some inwardly rectifying K^+ channels at the basolateral membranes in both amphibian (Mauerer et al. 1998a; Robson and Hunter 2005) and mammalian (Parent et al. 1988; Noulin et al. 1999; Ye et al. 2006) kidneys. They play a key role in the formation of cell negative potential, which serves as the driving force for several ions and solute transport across the tubular epithelia (Sackin and Boulpaep 1983). Thus, investigation of the regulatory mechanism for the K^+ channel is indispensable to clarifying the functional significance of proximal tubule not only in normal conditions but also in kidney diseases.

In cultured human renal proximal tubule epithelial cells (RPTECs), we have demonstrated that a native inwardly rectifying K^+ channel with inward conductance of about 40 pS is frequently observed in cell-attached patches under the control condition (Nakamura et al. 2002). Although the location of the K^+ channel in human proximal tubule cells

is still unknown, several lines of evidence suggest that the K^+ channel corresponds to the basolateral one. First, as mentioned above, the inwardly rectifying K^+ channels of the proximal tubule cells are mainly present at the basolateral membrane (Parent et al. 1988; Noulin et al. 1999; Ye et al. 2006). Second, the membrane potential of RPTECs is largely dependent on the activity of the K^+ channel (Nakamura et al. 2004), which is one of the important findings of the basolateral K^+ channel in the proximal tubule cell (Mauerer et al. 1998a). Third, the inwardly rectifying K^+ channel was not observed at the apical surface of confluently monolayered polarized RPTECs (Kubokawa et al., unpublished observation). Thus, it is likely that the inwardly rectifying K^+ channel with inward conductance of about 40 pS would correspond to the basolateral channel. However, ATP sensitivity, which is one of the significant characteristics of the basolateral K^+ channel in proximal tubule cells (Wang et al. 1997; Mauerer et al. 1998a), was not observed in the K^+ channel in RPTECs (Nakamura et al. 2002). Such a difference might result from alteration in channel composition induced by cell culture, although the ATP sensitivity of the human K^+ channel in situ is still unknown (Nakamura et al. 2002).

To date, it has been reported in the proximal tubule cells that protein kinase A (PKA) stimulates and protein kinase C (PKC) inhibits the activity of an inwardly rectifying K^+ channel at the basolateral membrane of salamander proximal tubule (Mauerer et al. 1998b). We have also demonstrated that the K^+ channel in RPTECs is activated by PKA and protein kinase G (PKG) (Nakamura et al. 2002). Thus, it is suggested that several kinds of protein kinases are involved in regulation of the inwardly rectifying K^+ channel in proximal tubule cells. In contrast to the several reports on protein kinase–mediated K^+ channel activity, only a few reports have shown the involvement of protein phosphatases in the regulation of K^+ channels in proximal tubule cells (Kubokawa et al. 2000). We have demonstrated that PKA-mediated activation of the K^+ channel in opossum kidney proximal tubule (OKP) cells is inhibited by protein phosphatases 1 (PP-1) and 2A (PP-2A), suggesting that PKA-mediated phosphorylation may be dephosphorylated by PP-1 or PP-2A (Kubokawa et al. 2000). As for the involvement of calcineurin (CaN), which is often called "protein phosphatase 2B," in regulation of the K^+ channel, Ye et al. (2006) demonstrated that one of CaN inhibitors, cyclosporin A (CsA), suppressed the basolateral K^+ channel in rat proximal tubule cells. However, the mechanism for the inhibitory effect of CsA on K^+ channel activity in this nephron segment has not been investigated.

Recently, we found that inhibitors of CaN—CsA and FK520, both of which are well-known immunosuppressive agents (Jan et al. 1999)—suppressed activity of the K^+ channel in RPTECs. Since CaN is known to induce the dephosphorylation of several phosphorylated protein serine/threonine residues (Donella-Deana et al. 1994; Rusnak and Mertz 2000), it is suggested that inhibition of CaN make some phosphorylation processes dominant. Thus, the channel inhibition mediated by inhibitors of CaN might result from protein phosphorylation. Based on such an idea, we investigated the effect of CaN on channel activity and proposed a role of CaN in the function of the K^+ channel in RPTECs.

Materials and Methods

Cell Culture

Secondary cultures of RPTECs from normal human kidneys were purchased from Lonza (Walkersville, MD). RPTECs were incubated in renal epithelial cell growth medium (REGM, Lonza) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. In the experiments, cells were isolated from 70–80% confluence at passages 3–6 with 0.025% trypsin and 0.01% EDTA and incubated on collagen-coated glass coverslips (Iwaki, Tokyo, Japan) with REGM for 3–10 h before use. The coverslip to which the cells adhered was then transferred into the control bath solution in a heating chamber (TC-324B, Warner, Hamden, CT). All experiments were performed at approximately 32°C, using the heating chamber.

Solutions

The pipette solution contained (mM) 145 KCl, 3 $MgCl_2$, 1 EGTA and 5 HEPES, pH adjusted to 7.3. The control bath solution contained (mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 glucose and 5 HEPES, pH adjusted to 7.3. The bath solution for inside–out patches contained (mM) 145 KCl, 3 $MgCl_2$ and 5 HEPES, with 10^{-6} M free Ca^{2+}. The solution with 10^{-6} M free Ca^{2+} was adjusted by the addition of an adequate amount of $CaCl_2$ and EGTA (10 mM) according to the computer program of Oiki and Okada (1987), which uses the absolute values of the stability constant of EGTA for the binding of Ca^{2+}, Mg^{2+} and H^+ (Martell and Smith 1977). Experimental bath solutions with pH adjusted to 7.3 were made by adding HCl or KOH. The free Ca^{2+} concentration and pH of all solutions were checked simultaneously with a Ca^{2+} electrode (CL-125B; TOA, Tokyo, Japan) and a pH electrode (GST-5311C, TOA), respectively, at approximately 33°C.

Patch-Clamp Technique

The patch-clamp technique was applied to single RPTECs, and channel recordings were performed in cell-attached or

excised inside–out patches. All patch-clamp experiments were performed at bath temperature of 32.0 ± 1.0°C. This setting of temperature was defined as the most appropriate for patch experiments using RPTECs (Nakamura et al. 2002). Patch-clamp electrodes were made of borosilicate glass capillaries (Warner) on a two-step puller (model PP-830; Narishige, Tokyo, Japan) and filled with the pipette solution described above. The electrode resistance ranged 3–5 MΩ. Electric currents measured with a patch-clamp amplifier (AXOPATCH 200B; Axon, Foster City, CA) were stored on a VHS tape recorder (RD-120TE; Toshiba, Tokyo, Japan). The current records were then low-pass-filtered (3611 Multifunction Filter; NF Electronic Instruments, Tokyo, Japan) at 500 Hz and digitized at a rate of 2.5 kHz through an interface (Digidata 1440A; Molecular Devices, Sunnyvale, CA). The acquired data were analyzed with pCLAMP8 software (Molecular Devices). Current traces of downward deflections represented inward currents.

Channel activity was determined by NP_o, which was calculated from an amplitude histogram as

$$NP_o = \sum_{n=1}^{N} n - t_n \quad (1)$$

where N is the maximum number of channels observed in the patch, P_o is the open probability, n is the number of channels observed at the same time and t_n is the probability that n channels are simultaneously open. Since the control values of NP_o varied among patches, we calculated normalized channel activity ($NP_{o,e}/NP_{o,c}$) for the convenient evaluation of effects of the substances. $NP_{o,c}$ and $NP_{o,e}$ are the channel activities under control and experimental conditions, respectively. Routinely, we determined $NP_{o,c}$ from a 20-s sampling period just before adding the substance when the steady state lasted for at least 40 s. $NP_{o,e}$ was determined from a 10-s sampling period extracted from the steady state for at least 20–30 s made by the experimental substance.

$[Ca^{2+}]_i$ Measurement

RPTECs were loaded with 10 μM fura-2-AM (Dojindo, Kumamoto, Japan) for 60 min at 37°C. After loading, the cells were thoroughly washed with the control bath solution at least three times to remove the excess dye and placed in a perfusion chamber mounted on a fluorescent microscope. Intracellular calcium ($[Ca^{2+}]_i$) measurements were carried out using a ratiometric imaging system (InCyt Basic IM; Intracellular Imaging, Cincinnati, OH), including a PC, a filter wheel of conventional design, a charge-coupled device camera and the TE 300 microscope (Nikon, Tokyo, Japan). In each experiment, a number of single cells was selected using the software. The fluorescent emissions as paired signals, at a wavelength of 510 nm from the selected regions, were measured according to excitation wavelengths of 340 and 380 nm at a time interval of 4 s.

Standard calibration of single RPTECs was carried out, and $[Ca^{2+}]_i$ was calculated using the equation described by Grynkiewicz et al. (1985) as

$$\left[Ca^{2+}\right]_i = K_d \cdot Q \cdot \frac{(R - R_{min})}{(R_{max} - R)} \quad (2)$$

where R represents the fluorescence intensity ratio $F_{\lambda 1}/F_{\lambda 2}$, in which $\lambda 1$ (∼ 340 nm) and $\lambda 2$ (∼ 380 nm) are the fluorescence detection wavelengths; R_{min} and R_{max} are the ratios corresponding to the minimum and maximal Ca^{2+} concentrations; Q is the ratio of F_{min} to F_{max} at $\lambda 2$ (∼ 380 nm); and K_d is the Ca^{2+} dissociation constant of the indicator. R_{max} was obtained by adding a Ca^{2+} ionophore, ionomycin (5 mM), to the control bath solution with 1 mM Ca^{2+}, and R_{min} was obtained by adding the ionomycin to the Ca^{2+}-free bath solution with 5 mM EGTA. The K_d value of 230 nM for fura-2 was adopted in this study.

The control value of $[Ca^{2+}]_i$ in individual experiments was determined as the mean value at the period for 30-s sampling data just before adding the test substance, and the experimental data were obtained from the mean value for 20-s data just before removal of the substance. In the statistical analyses, the relative changes in $[Ca^{2+}]_i$ in response to the test substances were obtained by comparing the experimental data ($[Ca^{2+}]_{i,e}$) with the control data ($[Ca^{2+}]_{i,c}$) of each cell. The data were expressed as $[Ca^{2+}]_{i,e}/[Ca^{2+}]_{i,e}$.

Western Blot

RPTECs were lysed using RIPA buffer (Pierce, Rockford, IL) containing a serine/threonine protein phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). An equivalent volume of Laemmli sample buffer (Bio-Rad, Hercules, CA) was added to the supernatants obtained by centrifugation (12,000 × g, 15 min) of cell lysates, and then the supernatant was boiled for 5 min at 100°C. Samples were subjected to SDS-PAGE on 10% polyacrylamide gels (Bio-Rad) and subsequently transferred to PVDF membrane. PVDF membranes were blocked with 5% nonfat milk (Cell Signaling, Beverly, MA) in TBS containing 0.1% Tween 20, then incubated overnight at 4°C with primary antibody (1:1,000). After incubation with horseradish peroxidase–linked secondary antibody (1:2,000; Cell Signaling) at room temperature for 60 min, immunoreactive bands were visualized with Amersham

ECL Plus detection reagents (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions.

Test Substances

The inhibitors of CaN CsA and FK520, the inhibitor of PKC GF109203X and CaN were purchased from Calbiochem (La Jolla, CA). Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and calmodulin (CaM) were purchased from UpState (Lake Placid, NY), and Na_2ATP was from Sigma. A CaMKII inhibitor, KN62, was purchased from Wako Pure Chemicals (Osaka, Japan). A fluorescent probe for Ca^{2+}, Fura-2-AM, was obtained from Dojindo. CsA, FK520 and KN62 were dissolved in dimethyl sulfoxide (DMSO) as stock solutions, whereas the others were dissolved in water. These stock solutions were diluted with the control bath solution before use and added to the bath by hand pipetting. The final concentration of DMSO in the bath ranged 0.02–0.06%, which did not affect channel activity. For Western blot analyses, CaMKII antibody and phospho-CaMKII (Thr286) antibody as primary antibodies were purchased from Cell Signaling.

Statistics

Data are expressed as means ± SE. Statistical significance was determined using Student's *t*-test or ANOVA in conjunction with the Bonferroni *t*-test. $P < 0.05$ was considered significant.

Results

An inwardly rectifying K^+ channel with inward conductance of about 40 pS is the K^+ channel which is most frequently observed using the patch-clamp technique in RPTECs under the control condition. The properties of the 40-pS K^+ channel have been demonstrated in our previous report in detail (Nakamura et al. 2002)

Effects of CaN Inhibitors on Activity of the Inwardly Rectifying K^+ Channel in RPTECs

To investigate the involvement of CaN in the modulation of the inwardly rectifying K^+ channel in RPTECs, we first applied inhibitors of CaN, CsA and FK520, an analogue of the well-known immunosuppressive agent FK506 (Baughman et al. 1995), to RPTECs to examine the effects of the inhibitors on channel activity in cell-attached mode of the patch-clamp technique. As shown in Fig. 1a, application of CsA (5 μM) markedly suppressed activity of the K^+ channel in RPTECs, and gradual restoration of channel activity was observed after removal of CsA. Similarly, as shown in Fig. 1b, FK520 (5 μM) also suppressed channel activity, and the suppressed activity could be restored after removal of the chemical. It took about 30–60 s to recover channel activity with both inhibitors. Summarized data of the effect of CsA and FK520 on channel activity are shown in Fig. 1c. Both CsA and FK520 significantly lowered channel activity compared with the control.

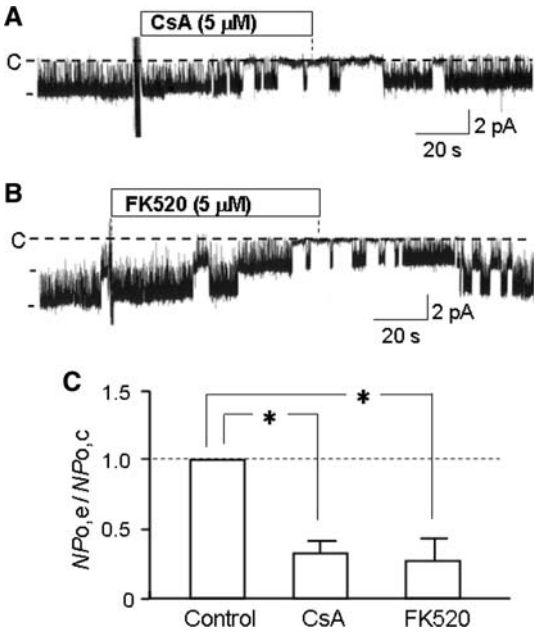

Fig. 1 Typical current recordings showing the effects of CaN inhibitors CsA and FK520 on activity of the inwardly rectifying K^+ channel in RPTECs. **a** Changes in channel activity in response to application of CsA at 5 μM to the bath solution. **b** Channel suppression induced by application of FK520 at 5 μM. Individual current recordings were obtained by separate cell-attached patches at a holding potential of 0 mV in the control bath solution. Closed channel states are indicated by dotted lines and C, and individual current levels of the K^+ channel are indicated by short bars (*left*). Both CsA and FK520 markedly suppressed channel activity. **c** Summarized data of effects of CsA ($n = 9$) and FK520 ($n = 8$) on changes in channel activity. Data are expressed as normalized channel activity ($NP_{o,e}/NP_{o,c}$) as described in "Materials and Methods." Both CsA and FK520 significantly suppressed channel activity compared to the control. $^*P < 0.05$

Changes in $[Ca^{2+}]_i$ in Response to Inhibitors of CaN

It has been reported that CsA or FK506, a well-known immunosuppressive agent, elevates $[Ca^{2+}]_i$ via release of Ca^{2+} from intracellular stores (Cameron et al. 1995; Bandyopadhyay et al. 2000; Bultynck et al. 2003). Thus, we next examined the effects of CsA and FK520, an analogue of FK506, on $[Ca^{2+}]_i$ of RPTECs using the fluorescent imaging of Fura-2. Figure 2 demonstrates the traces of the change in $[Ca^{2+}]_i$ in response to the application of CsA and FK520. The value of $[Ca^{2+}]_i$ was moderately elevated after addition of CsA or FK520. Summarized data are shown in

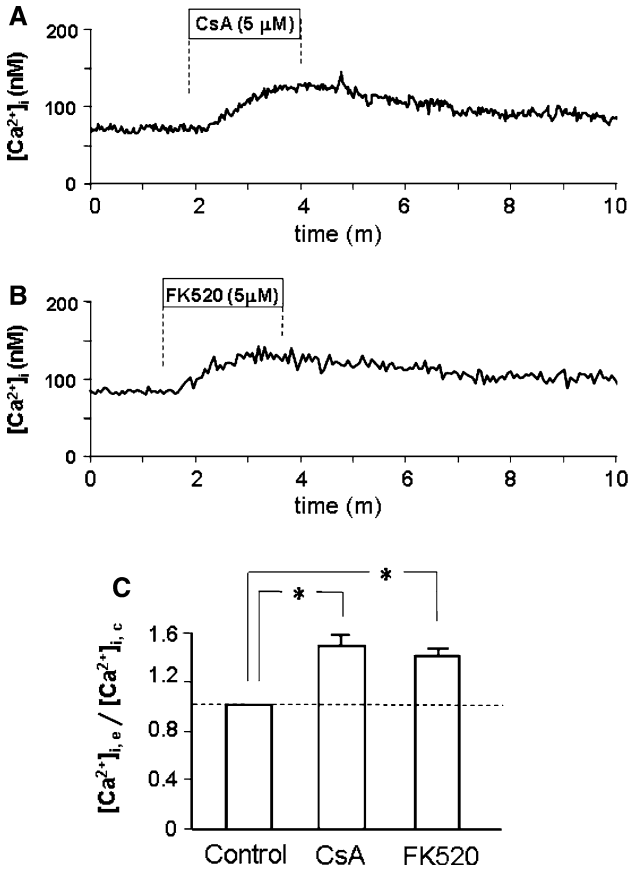

Fig. 2 Effects of CaN inhibitors on relative values of $[Ca^{2+}]_i$ estimated by the ratio of fluorescence at 340 and 380 nm (F340/F380). **a** Changes in $[Ca^{2+}]_i$ in response to addition of 5 μM CsA to the bath. **b** Effect of 5 μM FK520 on the relative values of $[Ca^{2+}]_i$. Both CsA and FK520 moderately elevated $[Ca^{2+}]_i$. **c** Summarized data of effects of CsA ($n = 10$) and FK520 ($n = 12$) on $[Ca^{2+}]_i$. Individual data were obtained about 2 min after addition of each substance, and the data were compared to the control and expressed as the ratio of experimental and control data (Re/RC), as described in "Materials and Methods." Both CsA and FK520 significantly elevated $[Ca^{2+}]_i$. $^*P < 0.05$

Fig. 2c, in which the value of $[Ca^{2+}]_{i,c}$ was set to 1. The values of $[Ca^{2+}]_{i,e}/[Ca^{2+}]_{i,c}$ in CsA (1.53 ± 0.09, $n = 19$) and FK520 (1.43 ± 0.05, $n = 20$) were both significantly increased compared to the control.

Since CaN is known to induce dephosphorylation of some proteins, the inhibitors of CaN suppress the dephosphorylation process, which would make phosphorylation dominant. Moreover, it was conceivable that elevated $[Ca^{2+}]_i$ by inhibitors of CaN would stimulate some Ca^{2+}-dependent protein phosphorylation. If this is the case, the phosphorylation might result in suppression of K^+ channel activity. Based on such an idea, we conducted the next experiment. Since the responses of channel activity and $[Ca^{2+}]_i$ to CsA and FK520 were quite similar, we adopted CsA as the inhibitor of CaN in the following experiments.

Effects of Inhibitors of PKC and CaMKII on CsA-Induced K^+ Channel Suppression

Among several agents which stimulate Ca^{2+}-dependent phosphorylation, PKC and CaMKII are the major candidates. Indeed, some reports have shown that activity of inwardly rectifying K^+ channels in renal tubule cells was inhibited not only by PKC (Wang and Giebisch 1991) but also by CaMKII (Kubokawa et al. 1995). Thus, we examined the effect of the CaN inhibitor CsA on channel activity in the presence of a PKC inhibitor, GF109203X, or a CaMKII inhibitor, KN-62, in the cell-attached patches. The results are shown in Fig. 3. GF109203X (500 nM) alone produced no remarkable change in channel activity, and the subsequent addition of CsA (5 μM) induced channel inhibition in the presence of GF109203X (Fig. 3a). KN-62 (20 μM) alone also did not affect channel activity but markedly abolished CsA-induced channel suppression (Fig. 3b). The summarized data are shown Fig. 3c. Application of GF109203X alone or KN-62 alone did not affect the channel activity, but addition of CsA significantly suppressed channel activity in the presence of GF109203X. In contrast, CsA-induced channel suppression was abolished by KN-62, suggesting that the CsA-induced channel suppression was mainly induced by the CaMKII-dependent processes.

Changes in $[Ca^{2+}]_i$ in Response to CsA in the Presence of a PKC Inhibitor or a CaMKII Inhibitor

It has been demonstrated that activity of an inwardly rectifying K^+ channel in amphibian proximal tubule cells was inhibited directly by elevation of intracellular Ca^{2+} (Mauerer et al. 1998a). Moreover, CaMKII was reported to facilitate Ca^{2+} release from intracellular Ca^{2+} stores (Tavi et al. 2003). Thus, it was possible that the CaMKII inhibitor KN-62 prevented the elevation of $[Ca^{2+}]_i$ by inhibiting CaMKII-mediated Ca^{2+} release, which might result in the prevention of CsA-induced channel inhibition. To examine whether KN-62 affects the CsA-induced elevation of $[Ca^{2+}]_i$ in RPTECs, we observed the effect of CsA on $[Ca^{2+}]_i$ in the presence of KN-62 using the fluorescent imaging of fura-2. Changes in $[Ca^{2+}]_i$ in the presence of a PKC inhibitor, GF109203X, were also examined to compare the effect of KN-62 with that of GF109203X on the CsA-induced change in $[Ca^{2+}]_i$.

Figure 4 shows the effect of GF109203X (500 nM) or KN-62 (20 μM) on CsA-induced $[Ca^{2+}]_i$ elevation. CsA (5 μM) was added about 2 min after addition of each inhibitor. Data on individual inhibitors were obtained from the mean value of a 30-s sampling period just before addition of CsA, and the value of CsA-induced change was obtained from a 30-s sampling period about 2 min after

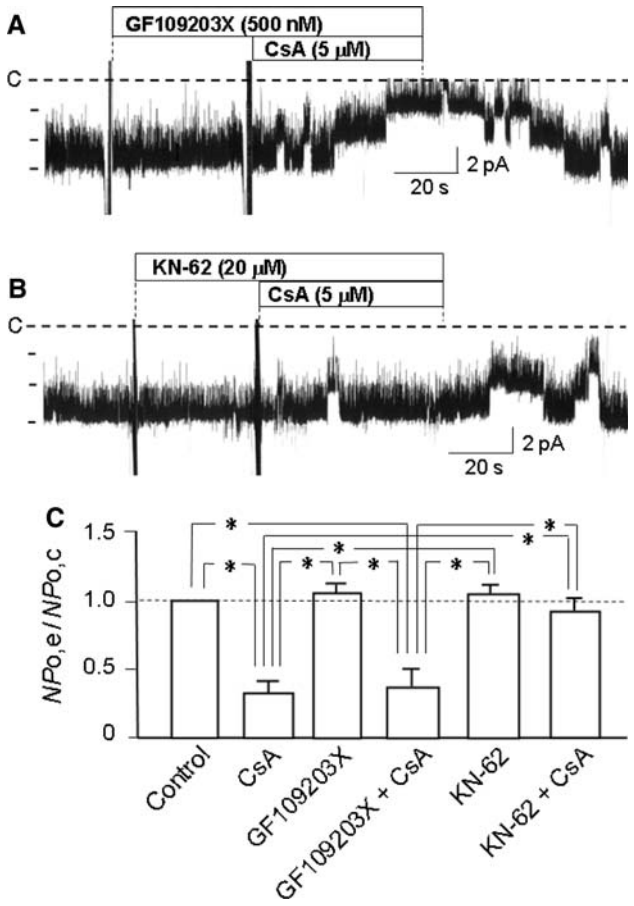

Fig. 3 Changes in CsA-induced channel suppression in the presence of PKC inhibitor GF109203X and CaMKII inhibitor KN-62. **a** Effects of GF109203X (500 nM) on channel activity before and after application of CsA (5 μM). GF109203X induced no appreciable change in channel activity, and subsequent addition of CsA suppressed channel activity. **b** Effects of KN-62 (20 μM) before and after application of CsA on channel activity. KN-62 did not affect channel activity, but the CsA-induced channel suppression was markedly attenuated in the presence of KN-62. Both traces were obtained in cell-attached patches at a holding potential of 0 mV. Closed channel states are indicated by dotted lines and C, and individual current levels of the K^+ channel are indicated by short bars (*left*). **c** Summarized data of channel activity in response to GF109203X ($n = 7$), GF109203X with CsA ($n = 7$), KN-62 ($n = 8$) and KN-62 with CsA ($n = 8$). Addition of GF109203X alone, KN-62 alone and KN-62 with CsA did not significantly alter the channel activity compared to the control, but CsA significantly suppressed channel activity even in the presence of GF109203X. $^*P < 0.05$

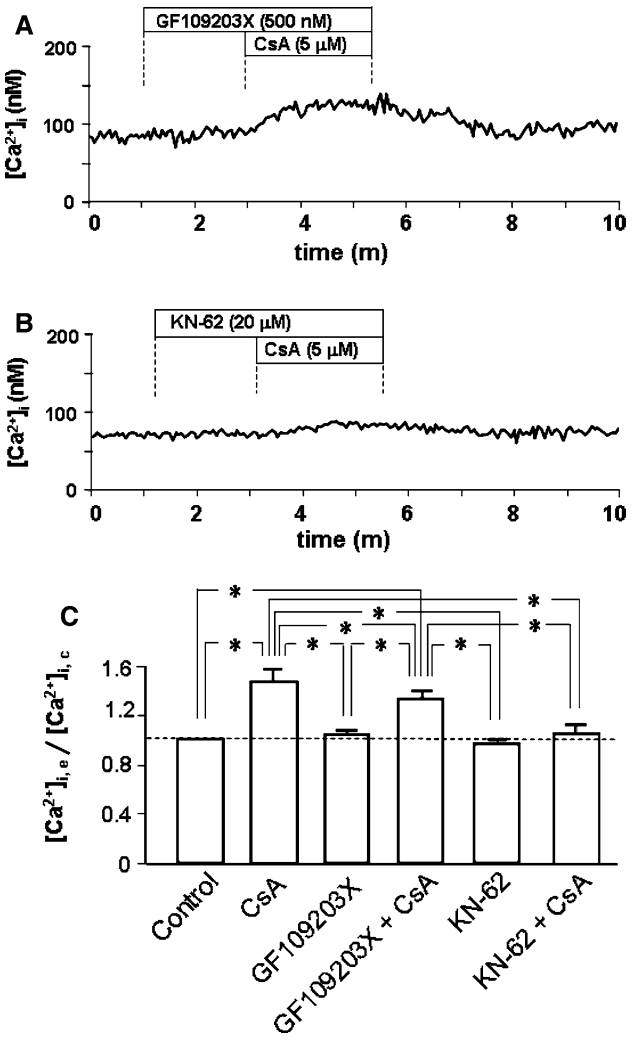

Fig. 4 Effects of GF109203X and KN-62 on CsA-induced $[Ca^{2+}]_i$ elevation. **a** Changes in $[Ca^{2+}]_i$ in response to CsA (5 μM) in the presence of GF109203X (500 nM). **b** CsA-induced changes in $[Ca^{2+}]_i$ in the presence of KN-62 (20 μM). GF109203X alone and KN-62 alone did not affect $[Ca^{2+}]_i$. CsA-induced $[Ca^{2+}]_i$ elevation was observed even in the presence of GF109203X but was prevented by the presence of KN-62. **c** Summarized data of effects of GF109203X alone and CsA with GF109203X on $[Ca^{2+}]_i$ ($n = 8$) and effects of KN-62 alone and CsA with KN-62 on $[Ca^{2+}]_i$ ($n = 12$). Individual data were obtained from mean values of a 60-s sampling period about 1 min after addition of GF109203X or KN-62 and about 2 min after addition of CsA, and the data were summarized as the ratio of experimental and control values (Re/RC). GF109203X alone and KN-62 alone induced no significant change in $[Ca^{2+}]_i$ compared to the control. CsA significantly elevated $[Ca^{2+}]_i$ in the presence of GF109203X ($n = 12$). $^*P < 0.05$

addition of CsA. As shown in Fig. 4a, CsA gradually elevated $[Ca^{2+}]_i$ even in the presence of GF109203X. However, as shown in Fig. 4b, CsA did not induce marked elevation of $[Ca^{2+}]_i$ in the presence of KN-62. The summarized data obtained by the experiments similar to Fig. 4a, b are shown in Fig. 4c. Normalized $[Ca^{2+}]_i$ ($[Ca^{2+}]_{i,e}/[Ca^{2+}]_{i,c}$) was not significantly affected by GF109203X alone (1.02 ± 0.02, $n = 17$) or by KN-62 alone (0.97 ± 0.01, $n = 16$). However, the subsequent addition of CsA significantly elevated $[Ca^{2+}]_i$ in the presence of GF109203X (1.32 ± 0.04, $n = 17$). In contrast, CsA did not significantly change $[Ca^{2+}]_i$ in the presence of KN-62 (1.03 ± 0.03, $n = 16$). These data appear to support the speculation that an elevation of $[Ca^{2+}]_i$ plays a crucial role in the CsA-induced channel suppression.

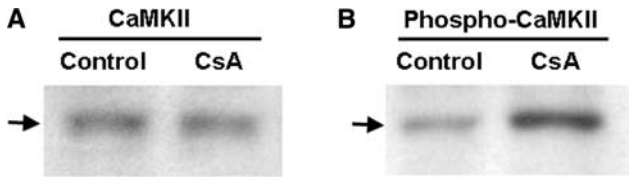

Fig. 5 Effects of CsA on CaMKII and phospho-CaMKII (Thr286) in RPTECs. **a** Western blot showing the effect of CsA (5 μM) on CaMKII detected with CaMKII antibody. CsA induced no appreciable change in CaMKII protein in RPTECs. **b** Western blot showing the effect of CsA (5 μM) on phospho-CaMKII detected with specific phospho-CaMKII antibody (Thr286). CsA obviously increased 50 kDa of phospho-CaMKII (Thr 286)

Detection of CaMKII and Phospho-CaMKII (Thr286) in RPTECs

Since the elevation of $[Ca^{2+}]_i$ could activate intracellular CaMKII, we investigated the changes in CaMKII protein and phospho-CaMKII (Thr286), an active form of CaMKII (Means 2000), with Western blot analysis.

Western blots of CaMKII and phospho-CaMKII (Thr286) in the absence or presence of CsA are shown in Fig. 5. In the case of presence of CsA, CsA (5 μM) was added to the control medium 2 min before starting Western blot analysis. CaMKII protein detected with CaMKII antibody was not influenced by CsA (Fig. 5a). Phospho-CaMKII (Thr286, 50 kDa) detected with a specific antibody of phospho-CaMKII, was obviously increased by CsA, although the band of phospho-CaMKII was slightly recognized in the control. These results indicate that total CaMKII in RPTECs was not affected by CsA but the active form of CaMKII, phospho-CaMKII (Thr286), was increased by CsA. Thus, it is likely that CsA-induced channel suppression resulted from stimulation of CaMKII-mediated protein phosphorylation.

Effects of Rise in $[Ca^{2+}]_i$ on Channel Activity in the Absence and Presence of Inhibitors of PKC and CaMKII

As indicated above, CsA-induced channel suppression required activation of CaMKII. However, it is still unknown whether activation of CaMKII was induced by elevation of $[Ca^{2+}]_i$ or application of CsA. To test whether the elevation of $[Ca^{2+}]_i$ without CsA is able to activate CaMKII, we adopted Ca^{2+} ionophore, ionomycin (1 μM), in the presence of 10^{-6} M Ca^{2+} in the bath. The values of $[Ca^{2+}]_i$ in response to ionomycin were measured with the fluorescent imaging of Fura-2. Although the traces of $[Ca^{2+}]_i$ in response to ionomycin are not shown, addition of ionomycin to the bath with 10^{-6} M Ca^{2+} remarkably increased $[Ca^{2+}]_i$, from 82.9 ± 4.9 to 317.0 ± 30.1 nM ($n = 11$). These values were significantly higher than the values of $[Ca^{2+}]_i$ elevated by CsA. The level of $[Ca^{2+}]_i$ was not altered in the presence of GF109203X and/or KN-62.

The effect of addition of ionomycin (1 μM) to the bath with 10^{-6} M Ca^{2+} on channel activity was examined in cell-attached patches. Ionomycin markedly suppressed channel activity in the absence of protein kinase inhibitors (Fig. 6-a). As observed in this trace, a spike current larger than the current of the 40-pS K^+ channel sometimes appeared after addition of ionomycin. Since ionomycin markedly increased $[Ca^{2+}]_i$, the current spikes would be produced by Ca^{2+}-activated BK channels in RPTECs (Kubokawa et al. 2005). We next applied the PKC inhibitor GF109203X (500 nM) or the CaMKII inhibitor KN-62 (20 μM) before addition of ionomycin to examine whether PKC or CaMKII was involved in the ionomycin-induced channel suppression. Each inhibitor was applied to the bath solution about 1 min before addition of ionomycin. The ionomycin-induced channel suppression was observed even in the presence of GF109203X (500 nM) or KN-62 (20 μM) (Fig. 6b, c). However, as shown in Fig. 5d, simultaneous application of KN-62 and GF109203X inhibited the ionomycin-induced channel suppression. The summarized data are shown in Fig. 6d. The results indicate that the marked elevation of $[Ca^{2+}]_i$ significantly suppressed channel activity even in the presence of GF109203X alone or KN-62 alone but failed to suppress the activity when both KN-62 and GF109203X were simultaneously present in the bath. Thus, these data suggested that the marked elevation of $[Ca^{2+}]_i$ suppressed channel activity by stimulation of both PKC and CaMKII. In other words, it is suggested that activation of CaMKII could be induced by elevation of $[Ca^{2+}]_i$, although the direct effect of CsA on CaMKII activation is still unknown.

Direct Effects of Cytoplasmic Ca^{2+} and CaM on Channel Activity in Inside–Out Patches

Despite the above data, a previous report showed that activity of an inwardly rectifying K^+ channel in proximal tubule cells was directly inhibited by internal Ca^{2+} in inside–out patches (Mauerer et al. 1998a). Thus, we further examined the direct effect of cytosolic Ca^{2+} on channel activity in inside–out patches. Moreover, some of the channel current was significantly affected by cytosolic CaM (Rycroft and Gibb 2004). Since cytosolic Ca^{2+} and CaM were necessary to stimulate both CaN and CaMKII, we investigated the direct effect of Ca^{2+}, as well as CaM, on channel activity in inside–out patches before investigating the direct effects of CaN and CaMKII. The K^+ channel with inward conductance of about 40 pS in RPTECs required 1 mM ATP to maintain its activity in inside–out patches, as reported previously (Nakamura et al. 2002). Thus, the following experiment in inside–out patches was carried out with 1 mM ATP in the bath solution.

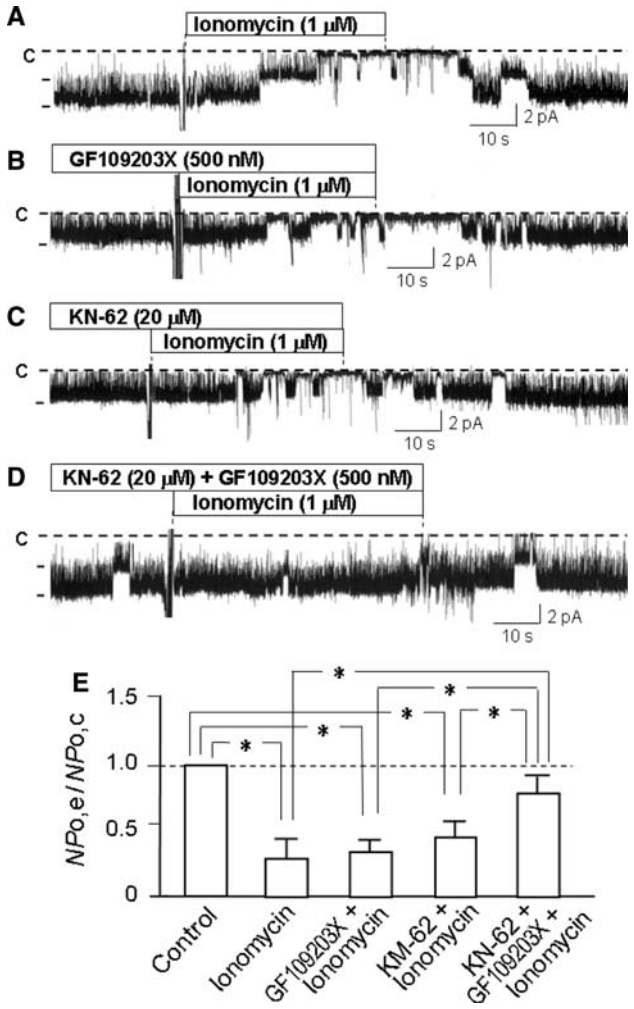

Fig. 6 Changes in channel activity in response to elevated $[Ca^{2+}]_i$ in the presence of protein kinase inhibitors in RPTECs. **a** Channel suppression induced by a Ca^{2+} ionophore, ionomycin (1 μM). The observed spike current other than the current of the inwardly rectifying K^+ channel after application of ionomycin was probably produced by Ca^{2+}-dependent components. **b** Channel inhibition induced by ionomycin in the presence of the PKC inhibitor GF109203X (500 nM). **c** Channel inhibition by ionomycin (1 μM) in the presence of the CaMKII inhibitor KN-62 (20 μM). **d** Prevention of ionomycin-induced channel suppression in the presence of KN-62 and GF109203X. Cells were treated with these inhibitors for more than 1 min before addition of CsA. Ionomycin-induced channel suppression was almost completely abolished by simultaneous pretreatment of cells with KN-62 and GF109203X. Traces were obtained in cell-attached patches at a holding potential of 0 mV. Closed channel states are indicated by dotted lines and C, and the current levels of the K^+ channel are indicated by short bars (*left*). **e** Summarized data of ionomycin (1 μM)-induced channel suppression in the absence and presence of inhibitors of CaMKII (KN-62, 20 μM) and PKC (GF109203X, 500 nM) at bath Ca^{2+} of 10^{-6} M. Channel activity was significantly suppressed by ionomycin ($n = 5$) even in the presence of GF109203X ($n = 6$) or KN-62 ($n = 6$). However, the channel suppression was significantly abolished when KN-62 and GF109203X were simultaneously present in the bath ($n = 6$). $^*P < 0.05$

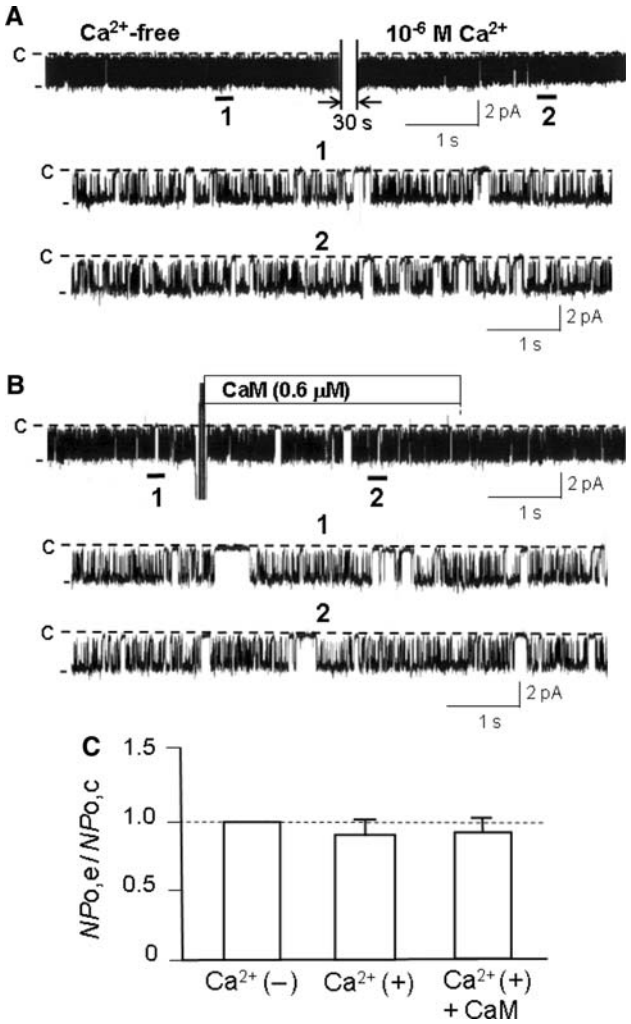

Fig. 7 Direct effects of Ca^{2+} and CaM on activity of the inwardly rectifying K^+ channel in RPTECs. **a** The effect on channel activity of replacement of Ca^{2+}-free bath with 10^{-6} M Ca^{2+}. The channel recording was made with in an inside–out patch in the presence of 1 mM ATP in the bath, and the holding potential of the patch pipette was +50 mV in a symmetrical KCl solution. Closed channel states are indicated by dotted lines and C, and open states of the channel are indicated by short bars (*left*). Two parts of the recordings indicated by short bars (1 and 2) in the *top trace* of **a** are displayed in the expanded time course below. **b** The direct effect of CaM (0.6 μM) from inside of the patch membrane on channel activity with 1 mM ATP and 10^{-6} M Ca^{2+} in the bath. The pipette holding potential was the same as above. Two parts of the recordings indicated by short bars (1 and 2) in the *top trace* of **b** are displayed in the expanded time course below. **c** Summarized data of effects of 10^{-6} M Ca^{2+} ($n = 5$) and CaM ($n = 5$) on channel activity. Ca^{2+}-free and 10^{-6} M Ca^{2+} were abbreviated to Ca^{2+} (–) and Ca^{2+} (+), respectively. Channel activity at Ca^{2+} (–) was set to 1.0

Representative current traces in response to replacement of the bath with Ca^{2+}-free to 10^{-6} M Ca^{2+} in an inside–out patch are shown in Fig. 7a. Two parts of the recordings,

indicated by short bars (1 and 2) in the top trace, are displayed in the expanded time course below. In this case, the values of P_o obtained from 20-s data around the numbered parts before and after addition of Ca^{2+} were 0.70 and 0.67, respectively. Furthermore, as shown in Fig. 7b, the effect of direct application of CaM (0.6 mM) to the cytoplasmic site of the patch membrane was also examined in the inside–out patch. Two parts of the recordings, indicated by short bars (1 and 2) in the top trace of Fig. 7b, are displayed in the expanded time course below. The values of P_o obtained from 20-s data before and after addition of CaM were 0.60 and 0.58, respectively. The results are summarized in Fig. 7c. From these data, we confirmed that Ca^{2+} and CaM had no direct effect on channel activity. The following experiments in inside–out patches were carried out with 10^{-6} M Ca^{2+} in addition to 1 mM ATP in the bath.

Direct Effects of CaN and CaMKII on Channel Activity in Inside–Out Patches

Current traces of direct application of cytoplasmic CaN and CaMKII after addition of CaM in the presence of 1 mM ATP and 10^{-6} M Ca^{2+} are shown in Fig. 8. Application of CaN (800 U/ml) to the cytoplasmic side of the patch membrane for about 1 min induced no remarkable change in channel activity. On the other hand, addition of CaMKII (0.15 U/ml) to the bath suppressed channel activity. The channel suppression was usually observed in 1 min. Data are summarized in Fig. 8c. Although CaN produced statistically no significant change in channel activity, CaMKII significantly suppressed the activity.

In the final series of experiments, CaM, CaN and CaMKII were successively applied in inside–out patches in the presence of 10^{-6} M Ca^{2+} and 1 mM ATP. Representative current traces of changes in channel activity in response to CaM, CaN and CaMKII are shown in Fig. 8a, b. Application of CaN (800 U/ml) after addition of CaM (0.6 μM) induced no appreciable change in channel activity, which is similar to the result shown in Fig. 8a, c. After we confirmed that the channel activity was stable, we added CaMKII (0.15 U/ml) to the bath to examine the effect of CaMKII on channel activity in the presence of CaM and CaN. As shown in Fig. 9a, CaMKII-induced channel suppression was almost completely abolished by the presence of CaN. Furthermore, we investigated the effect of CaN on CaMKII-induced channel suppression. Addition of CaMKII (0.15 U/ml) in the presence of CaM suppressed channel activity, and subsequent addition of CaN (0.15 U/ml) recovered its activity, as shown in Fig. 9b. This recovery was usually observed in 10–30 s. From the summarized data of Fig. 9c, although CaN did not induce significant change in channel activity, the presence of CaN abolished the CaMKII-mediated channel suppression. Moreover, even when channel activity was suppressed by CaMKII, CaN reactivated the suppressed channel to near the control level.

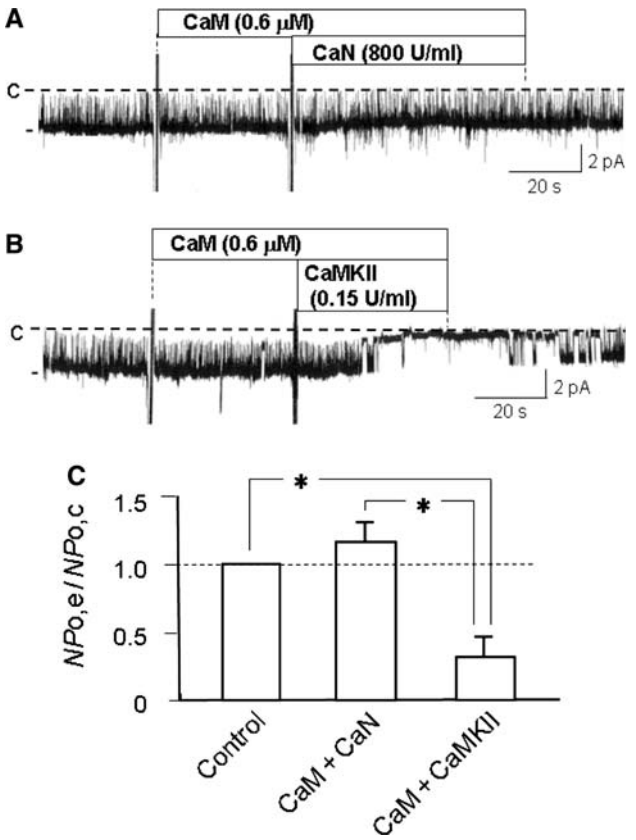

Fig. 8 Direct effects of CaN and CaMKII on channel activity. **a** A representative trace of the effect of CaN (800 U/ml) with CaM (0.6 μM) on channel activity in an inside–out patch in the presence of 10^{-6} M Ca^{2+} and 1 mM ATP in the bath. The holding potential of the patch pipette was +50 mV in a symmetrical KCl solution. Closed channel states are indicated by dotted lines and C, and open state of the channel are indicated by short bars (*left*). The inside of the patch membrane was exposed to CaN for more than 1 min, but no remarkable change was observed. **b** Direct effect of CaMKII (0.15 U/ml) on channel activity in an inside–out patch. The bath solution contained 0.6 μM CaM, 10^{-6} M Ca^{2+} and 1 mM ATP and the holding potential was the same as above. Application of CaMKII to the bath suppressed channel activity in 30 s, and removal of CaMKII led to slowly recovered channel activity. **c** Summarized data of direct effects of CaN ($n = 7$) and CaMKII ($n = 8$) on channel activity. Although CaN moderately raised channel activity, no significant difference was obtained compared to control. In contrast, direct application of CaMKII significantly suppressed channel activity compared to control. $^*P < 0.05$

Discussion

The functional significance and molecular characteristics of CaN have widely been investigated in a variety of cells and tissues (Rusnak and Mertz 2000). CaN induces

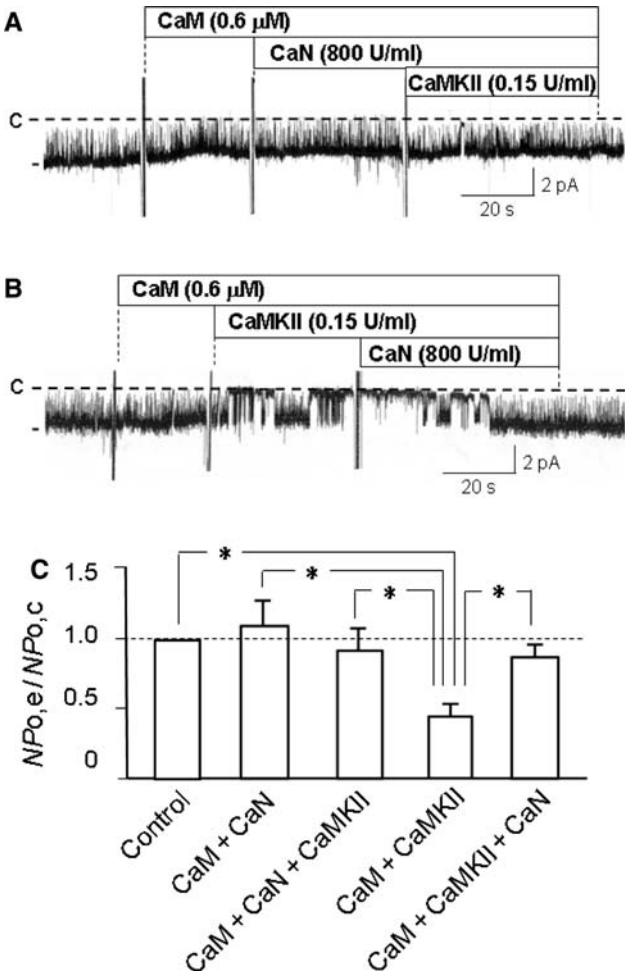

Fig. 9 Direct effects of CaN on CaMKII-induced changes in channel activity. **a** Prevention of CaMKII-induced channel suppression in the presence of CaN. **b** Suppression of channel activity induced by CaMKII (0.15 U/ml) and restoration of activity by subsequent addition of CaN (800 U/ml). Both traces were obtained in inside–out patches in the presence of CaM (0.6 μM), 10^{-6} M Ca^{2+} and 1 mM ATP. The holding potential of the patch pipette was +50 mV in a symmetrical KCl solution in both traces. Closed channel states are indicated by dotted lines and C, and open state of the channel are indicated by short bars (*left*). **c** Summarized data obtained from similar experiments as shown in **a** and **b**. CaMKII-induced channel suppression was abolished when CaN was present in the bath ($n = 6$), and CaMKII-induced channel suppression was significantly reactivated by CaN ($n = 5$). *$P < 0.05$

dephosphorylation of protein kinase–mediated phosphorylation (Donella-Deana et al. 1994) and plays several key roles in many cellular metabolisms. Among the abundant knowledge on CaN, an inhibitor of CaN, CsA, is well known as an immunosuppressive agent and is clinically used after organ transplantation or for therapy of autoimmune diseases. However, several complications, such as hypertension or hyperkalemia (Ventura et al. 1997), have been observed during clinical use of CsA (Myers et al. 1988; Borel 1991). It has been reported that CsA-induced complications were partly due to decreases in renal function (Myers et al. 1988; Borel 1991; Tumlin 1997). Ling and Eaton (1993) proposed using rabbit cortical collecting duct (CCD) to inhibit the apical K^+ channel, which induced a decrease in plasma K^+ secretion to urine through the K^+ channel, resulting in hyperkalemia. They suggested that inhibition of the apical K^+ channel was induced by stimulated PKC-mediated phosphorylation via a CsA-induced increase in $[Ca^{2+}]_i$. Indeed, the Ca^{2+}/PKC-dependent inhibition of the apical K^+ channel in CCD was previously demonstrated by Wang and Giebisch (1991). As for the elevation of $[Ca^{2+}]_i$, several reports have shown that inhibition of CaN induces receptor-mediated Ca^{2+} release from intracellular Ca^{2+} stores (Cameron et al. 1995; Bandyopadhyay et al. 2000; Bultynck et al. 2003) or CaN-mediated Ca^{2+} entry (Burley and Sihra 2000). On the other hand, Tumlin (1997) showed the importance of CaN in the signaling pathway of potassium metabolism in kidney cells such that the inhibition of CaN by CsA or FK506 induced nephrotoxic hyperkalemia. Recently, Zhang et al. (2008) also demonstrated that inhibition of CaN impairs renal K^+ secretion through the intracellular signaling pathway of CCD cells.

CaN Inhibitor-induced K^+ Channel Suppression and $[Ca^{2+}]_i$ Elevation

It has been reported that CaN is most abundant in the proximal tubule cells among several nephron segments (Tumlin 1997). Thus, CaN is an important factor for the function of proximal tubule cells. Ye et al. (2006) reported that CsA suppressed the activity of the inwardly rectifying K^+ channel in rat proximal tubule cells. We also found that inhibitors of CaN, CsA or FK520, suppressed K^+ channel activity in RPTECs (Fig. 1a, b). However, the mechanisms for the CsA-induced channel suppression in proximal tubule cells had not been investigated. In this study, we examined the mechanism for the CaN inhibitor–mediated channel suppression by observing changes in $[Ca^{2+}]_i$ and channel activity in response to CsA and FK520. Our $[Ca^{2+}]_i$ measurements showed that both CsA and FK520 increased $[Ca^{2+}]_i$ (Fig. 2a, b), which was similar to the data reported previously (Cameron et al. 1995; Bandyopadhyay et al. 2000; Bultynck et al. 2003). Thus, Ca^{2+}-dependent protein kinases, such as PKC, may be a key factor for the CaN inhibitor–mediated channel suppression, as proposed by Ling and Eaton (1993).

To examine the involvement of Ca^{2+}-dependent protein kinase in CsA-induced K^+ channel suppression, we tested the effect of a PKC inhibitor, GF109203X, and a CaMKII inhibitor, KN-62, on channel suppression. CsA-induced channel suppression was not affected by GF109203X but significantly attenuated by KN-62 (Fig. 3), suggesting that the suppression was mediated mainly by CaMKII. Although

PKC may have an inhibitory effect on the inwardly rectifying K^+ channel in renal tubule cells (Wang and Giebisch 1991), our results suggest that the major candidate which evokes CsA-induced channel suppression is CaMKII in RPTECs. This notion was supported by evidence that an active form of phospho-CaMKII was increased by CsA (Fig. 5), although it is unclear whether the CsA-dependent activation of CaMKII is directly induced by CsA or by elevation of $[Ca^{2+}]_i$, at least at this point.

It has also been demonstrated that CaMKII stimulates Ca^{2+} release from intracellular Ca^{2+} stores (Tavi et al. 2003). Our data that inhibition of CaMKII abolished the CsA-induced $[Ca^{2+}]_i$ elevation (Fig. 4) also support the report by Tavi et al. (2003). From these data, there remains a possibility that the CsA-induced channel suppression may be simply dependent on elevation of $[Ca^{2+}]_i$. In this regard, we further examined the effect of elevation of $[Ca^{2+}]_i$ on channel activity without CsA using the Ca^{2+} ionophore ionomycin. Although ionomycin induced channel suppression and the suppression was not blocked by the CaMKII inhibitor KN-62 alone or the PKC inhibitor GF109203X alone, simultaneous addition of KN-62 and GF109203X significantly abolished the channel suppression (Fig. 6). These data strongly suggested that the channel suppression induced by ionomycin which led to a marked elevation of $[Ca^{2+}]_i$ resulted from stimulation of both CaMKII and PKC. They is also suggested that elevation of $[Ca^{2+}]_i$ alone can activate CaMKII without CsA, although the direct effect of CsA on activation of CaMKII is still unknown.

Probably, a relatively low $[Ca^{2+}]_i$ would activate only CaMKII and a high level of $[Ca^{2+}]_i$ would activate PKC in addition to CaMKII. Moreover, we confirmed that intracellular Ca^{2+} did not directly affect channel activity in inside–out patches (Fig. 7a, c), indicating that CsA-induced channel suppression was not directly mediated by $[Ca^{2+}]_i$ but mainly induced by CaMKII. These findings suggest that the regulatory mechanism of the K^+ channel in RPTECs by Ca^{2+} and CaMKII is different from that of the basolateral K^+ channel in salamander kidney proximal tubule cells, reported by Mauerer et al. (1998b). Although the difference in Ca^{2+} sensitivty between the inwardly rectifying K^+ channel in salamander proximal tubule cells (Mauerer et al. 1998b) and the K^+ channel in RPTECs shown in this study is unknown, it may be caused by the difference of species or by the difference of isolated cells and cultured cells. On the other hand, regulatory processes of the K^+ channel in RPTECs were similar to those of the apical K^+ channel (ROMK1 or ROMK2 channel) of rat kidney CCD cells (Kubokawa et al. 1995). However, the K^+ channel in RPTECs is distinctly different from ROMK channels since the inward slope conductance and location of the two are different. Namely, the mean inward conductance of the K^+ channel in RPTECs is 42.5 pS (Nakamura et al. 2002), whereas that of ROMK, which is expressed only in the distal nephron but not in the proximal tubule, is about 30 pS (Wang et al. 1997).

Modulation of K^+ Channel Activity by CaN and CaMKII

Direct effects of CaN and CaMKII on channel activity were examined in inside–out patches. Before investigation of the effects of CaN and CaMKII, we tested direct effects of cytoplasmic Ca^{2+} and CaM on channel activity since it was reported that activity of some channels was directly affected by Ca^{2+} (Mauerer et al. 1998a) or CaM (Rycroft and Gibb 2004). As the result, we have confirmed that Ca^{2+} (10^{-6} M) or CaM (0.6 μM) hardly affects K^+ channel activity from the inside of the membrane in RPTECs (Fig. 7b, c). Application of CaN in the presence of Ca^{2+} and CaM also induced no appreciable change in channel activity (Fig. 8a, c). This seems to indicate that CaN has no direct effect on channel activity. In contrast, CaMKII markedly suppressed channel activity (Fig. 8b, c), suggesting that CaMKII-mediated phosphorylation has an inhibitory effect on channel activity. In the final experiments, we successively added CaMKII and CaN in inside–out patches and investigated their roles in the modulation of channel activity. The result indicates that CaMKII-mediated suppression was abolished in the presence of CaN (Fig. 8a, c). Furthermore, CaMKII-induced channel suppression was recovered by subsequent addition of CaN (Fig. 9b, c), suggesting that CaMKII-mediated channel suppression is inhibited by CaN-mediated dephosphorylation. Thus, CaN-mediated dephosphorylation would play an important role in supporting channel activity by preventing CaMKII-dependent processes.

However, channel modulation by CaN and CaMKII would not be simple since Ca^{2+} and CaM are common factors for activation of both CaN and CaMKII. It is conceivable that the power balance between phosphorylation by CaMKII and dephosphorylation by CaN would be important for alternating channel activity. In our experiments, inhibition of CaN in intact RPTECs moderately increased $[Ca^{2+}]_i$ (Fig. 2) and induced channel suppression which resulted from stimulation of CaMKII (Fig. 3). Although $[Ca^{2+}]_i$ was generally low as measured with Fura-2 imaging in intact RPTECs, these results suggest that CaN maintained a dephosphorylation state to maintain channel activity even in the control condition. Thus, CaN would be effective for protein dephosphorylation at low $[Ca^{2+}]_i$ conditions. In contrast, CaMKII-mediated phosphorylation was evoked when CaN was inhibited and $[Ca^{2+}]_j$ was moderately elevated, which resulted in channel suppression. Moreover, a further increase in $[Ca^{2+}]_i$

stimulated not only CaMKII but also PKC (Fig. 4). In such high $[Ca^{2+}]_i$ conditions, CaMKII- and PKC-dependent effects would exceed the CaN-dependent processes. Taken together, CaN-mediated phosphorylation is dominant in a condition with low $[Ca^{2+}]_i$, a moderate increase in $[Ca^{2+}]_i$ stimulates CaMKII and a further increase in $[Ca^{2+}]_i$ exceeds the effect of CaN by additional stimulation of PKC. Probably, the activity of these enzymes is regulated not only by intracellular Ca^{2+} and CaM but also by several other factors in intact cells which modulate channel activity.

A Possibility of a Functional Relationship Between CaN and CaMKII

Previous reports have shown that CaN plays several key roles in cellular function. As for the Ca^{2+} release from intracellular stores, CaN is involved in its regulation. For example, inhibition of CaN by FK506 enhanced Ca^{2+} release through the inositol 4,5-triphosphate receptor (IP3R) (Cameron et al. 1995), and CaN inhibitors lead to a higher open probability of the ryanodine receptor (RyR)/Ca^{2+}-release channel (Bandyopadhyay et al. 2000; Bultynck et al. 2003). On the other hand, CaMKII was reported to stimulate Ca^{2+} release in skeletal muscle cells (Tavi et al. 2003). Thus, it is likely that CaMKII-mediated stimulation of Ca^{2+} release was similar to the enhanced Ca^{2+} release induced by inhibition of CaN. Thus, CaMKII and CaN may have opposite effect on Ca^{2+} release from the intracellular Ca^{2+} store. These reports seem to support our experimental data that CaN and CaMKII have opposite effects on channel activity. However, it has been reported that the target phosphorylation site which was dephosphorylated by CaN is mainly mediated by PKA (Donella-Deana et al. 1994). Indeed, functional coupling of CaN and PKA was shown to modulate Ca^{2+} release in ventricular myocytes (Santana et al. 2002). It has also been demonstrated that Na^+/K^+-ATPase at the basolateral membrane of kidney tubular epithelia was inhibited by CaN (Tumlin and Sands 1993) and stimulated by PKA (Carranza et al. 1998). On the other hand, the cardiac Na^+/Ca^{2+} exchanger was reported to be regulated by CaN and PKC (Shigekawa et al. 2007). A cellular process which depends on mitogen-activated protein kinase was reported to be negatively regulated by CaN (Tian and Karin 1999). Thus, the protein kinases opposed to CaN-mediated processes would not be the same. Only a few reports have suggested that CaMKII-mediated processes were abolished by CaN (Wu et al. 2002; Gerges et al. 2005). Our data indicate a possibility that CaMKII-mediated phosphorylation was blocked by CaN.

Moreover, it is suggested that CaN-mediated protein dephosphorylation is dominant compared to CaMKII-mediated phosphorylation, at least in our control conditions, since CaN inhibitors significantly affected channel activity but an inhibitor of CaMKII alone did not. This observation was supported by the previous report that CaN content in proximal tubule cells is high among the nephron segments (Tumlin 1997). Although involvement of CaN in modulation of channel activity is varied (Lieberman and Mody 1994; Czirjak et al. 2004; Loane et al. 2006; Zhang et al. 2008; Wang et al. 2009), our data suggest that CaN plays an important role in supporting the activity of the inwardly rectifying K^+ channel by preventing the CaMKII-mediated phosphorylation processes in RPTECs in control normal conditions.

Conclusions

The involvement of CaN in modulating the activity of a native inwardly rectifying K^+ channel was investigated in RPTECs. We found that activity of the K^+ channel in RPTECs is supported at least in part by CaN, which is often called protein phosphatase 2B (PP-2B). CaN-mediated dephosphorylation has a stimulatory effect on channel activity by preventing CaMKII-dependent processes. It is also suggested that PKC inhibits the channel activity at a high $[Ca^{2+}]_i$ (Fig. 6). Previously, we demonstrated that cAMP-dependent PKA- and cGMP-dependent PKG-mediated phosphorylation stimulated channel activity in RPTECs (Nakamura et al. 2002). Considering these data, we conclude that at least two distinct phosphorylation sites, stimulatory and inhibitory, are present in the channel or associated protein and play a role in the regulation of channel activity. Moreover, since CaN-mediated dephosphorylation activates the channel against CaMKII-mediated suppression, maintenance of channel opening would require the CaN-mediated action to exceed the CaMKII-mediated action on the channel (Fig. 10). However, it is still unknown whether the site of CaN-mediated dephosphorylation is the same as that of CaMKII-mediated

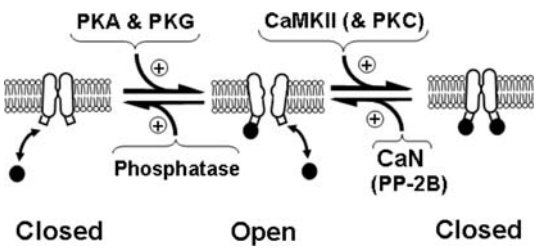

Fig. 10 A model for regulation of the inwardly rectifying K^+ channel in cultured renal proximal tubule cells by phosphorylation and dephosphorylation processes. CaN is often called protein phosphatase-2B (PP-2B). Closed circles indicate phosphate, and small squares attached to the lower part of the channel are putative phosphorylation sites. See text for details

phosphorylation in the modulation of channel activity. Further study will be necessary to clarify the molecular characteristics for regulating channel activity by the specific protein kinases and phosphatases.

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