Ca²⁺-Dependent Inhibition of Inwardly Rectifying K⁺ Channel in Opossum Kidney Cells

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Abstract: The effect of intracellular Ca²⁺ on the activity of the inwardly rectifying ATP-regulated K⁺ channel with an inward conductance of about 90 pS was examined by using the patch-clamp technique in opossum kidney proximal tubule (OKP) cells. The activity of the inwardly rectifying K⁺ channel rapidly declined with an application of ionomycin (1 μM) in the presence of 10^{−6} M Ca²⁺ in cell-attached patches. The application of 10 μM phorbol-12-myristate-acetate (PMA) with 10^{−6} M Ca²⁺ reduced the K⁺ channel activity. Although the channel activity was not influenced by an increase of bath Ca²⁺ from 10^{−7.5} to 10^{−6} M, the activity was inhibited by protein kinase C (PKC, 1 U/ml) with 10^{−6} M Ca²⁺ in inside-out patches. The inhibitory effect of Ca²⁺ with ionomycin on the channel activity was diminished by the pretreatment with a specific PKC inhibitor, GF 109203X (5 μM), in cell-attached patches. By contrast, the application of Ca²⁺/calmodulin kinase II (CaMK II, 300 pM) dramatically increased

this channel activity in inside-out patches. In cell-attached patches, the addition of both GF 109203X and cyclosporin A (5 μM), a potent inhibitor of protein phosphatase 2B (calcineurin), instead stimulated the K⁺ channel activity with ionomycin and 10^{−6} M Ca²⁺. The addition of protein phosphatase 2B (calcineurin) (2 U/ml) to the bath with calmodulin (1 μM) and Ni²⁺ (10 μM) to stimulate calcineurin inhibited the channel activity in inside-out patches. Furthermore, the inhibitory effect of PKC or calcineurin on this channel activity was abolished by a removal of Ca²⁺ from bath solution. These results suggest that Ca²⁺-dependent inhibitory effect on the inwardly rectifying K⁺ channel in OKP cells was mainly mediated by Ca²⁺-PKC-mediated phosphorylation, and that the Ca²⁺-calmodulin-dependent phosphorylation process may be counterbalanced by the Ca²⁺-calmodulin-dependent dephosphorylation process. [Japanese Journal of Physiology, 51, 371–380, 2001]

Key words: proximal tubule cell, inwardly rectifying K⁺ channel, PKC, CaMK II, calcineurin.

It has been demonstrated in both mammalian and amphibian proximal tubule cells with the patch-clamp and microelectrode techniques that the inwardly rectifying pH-sensitive and ATP-regulated K⁺ channel with a conductance of about 50 pS mediate the membrane potential of proximal tubule cells [1–3]. We have demonstrated that in cultured opossum kidney proximal tubule (OKP) cells, the inwardly rectifying pH-sensitive K⁺ channel with a conductance of about 70–90 pS is regulated by ATP, protein kinase A

(PKA), and inhibited by Ba²⁺ or glybenclamide, a specific inhibitor of sulfonylurea receptor [4–6]. Besides electrophysiological studies, the cloning and reconstitution studies demonstrated that ATP-sensitive K⁺ channels are heteromultimers of inwardly rectifying K⁺ channel subunits (Kir6.x) and sulfonylurea receptors (SUR) [7]. Moreover, it has also been indicated with the histochemical studies of the inwardly rectifying ATP-sensitive K⁺ channel (Kir6.1) that Kir6.1 with a conductance of 70 pS is ubiquitously

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distributed along the nephron and may be functionally related with Na^+ - K^+ ATPase along the renal tubule [8], suggesting that the inwardly rectifying K^+ channel in OKP cells is involved in the Kir6.x. All these studies support the view that the inwardly rectifying pH-sensitive and ATP-regulated K^+ channel in the basolateral membrane of the proximal tubular cell may participate in generating the cell-negative potential, which gives the driving force for electrogenic apical Na^+ entry and basolateral HCO_3^- (or Cl^-) exit.

Several investigators have reported that intracellular Ca^{2+} plays important roles in the regulation of K^+ channels of individual nephron segments [9]. In the principal cells of the rat cortical collecting duct (CCD), it has been demonstrated that the inhibition of a basolateral Na-K pump by a manipulation that raises intracellular Ca^{2+} reduces apical K^+ channel activity, and that Ca^{2+} -dependent protein kinase C (PKC) and Ca^{2+} /calmodulin-dependent protein kinase II (CaMK II) play a key role in mediating the regulation of K^+ channel activity [10, 11]. However, little is known about modulation of the channel activity in the proximal tubule cells by intracellular Ca^{2+} or Ca^{2+} -dependent processes.

Recently, Mauerer *et al.* have reported that the ATP-sensitive K^+ channel in the basolateral membrane of *Ambystoma* proximal tubule cells is activated by protein kinase A (PKA) and inhibited by ATP, Ca^{2+} , PKC, and cytosolic acidosis [12, 13]. In preliminary studies of OKP cells, we have also demonstrated that dual regulation of PKA and PKC to this inwardly rectifying pH-sensitive K^+ channel, i.e., the activation of the K^+ channel by PKA and its inhibition by PKC [14]. However, the effect of other Ca^{2+} -dependent processes, such as CaMK II, on this channel activity has not been elucidated.

In the present study, we investigated the regulation of the inwardly rectifying ATP-regulated K^+ channel by PKC, protein phosphatase-2B (calcineurin), and CaMK II, using the patch-clamp technique in OKP cells. We suggest that PKC-induced inhibition is a dominant Ca^{2+} -dependent process in the regulation of this K^+ channel activity.

MATERIALS AND METHODS

Cell culture. OKP cells were maintained in Dulbecco's modified Eagle's medium (Dainippon Pharmaceutical, Tokyo, Japan), supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 , as previously reported [4]. In these experiments, the cells were isolated from confluent monolayers at passage 51–106 with trypsin and incubated in the culture medium for

4 to 8 h before use. All experiments were performed at room temperature (approx. 24°C).

Solutions. The control bath solution contained (in mM) 140 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 5 glucose, and 10 N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES). In cell-attached patches, NaCl solutions with $10^{-7.5}$, 10^{-7} , $10^{-6.5}$, and 10^{-6} M free Ca^{2+} were used, which contained 110 NaCl, 5 KCl, 1 MgCl_2 , 30 NaOH, 10 ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and 5 HEPES with adequate free Ca^{2+} . The KCl solution used for bath in inside-out patches contained 115 KCl, 3 MgCl_2 , 30 KOH, 10 EGTA, and 5 HEPES with 10^{-8} , $10^{-7.5}$, 10^{-7} , $10^{-6.5}$, 10^{-6} , and 10^{-5} M free Ca^{2+} . The free Ca^{2+} concentration was obtained via the computer program of Oiki and Okada [15], which was made by using the absolute values of stability constant of EGTA for the binding of Ca^{2+} , Mg^{2+} , and H^+ [16–18]. The actual concentration of free Ca^{2+} was checked by using Ca^{2+} -selective microelectrodes [19] made with a Ca^{2+} -ion exchanger (ETH-1001, Science Trading, Frankfurt, Germany). The solutions with ATP were made by adding Na_2ATP (Nacalai Tesque, Kyoto, Japan). All these solutions were adjusted to pH 7.3 with a minute amount of HCl, NaOH, or KOH at 24°C.

Chemicals. Chemicals used were obtained from Calbiochem-Novabiochem (La Jolla, CA), except calcineurin (Upstate Biotechnology Inc., Lake Placid, NY) and albumin and nickel chloride (Wako Pure Chemicals, Tokyo, Japan). Stock solutions of ionomycin (250 μM), PMA (1 mM), and GF109203X (500 μM) were prepared in DMSO, which did not alone affect the K^+ channel activity in the concentration range of less than 0.1% used in the present experiments. The stock solution of cyclosporin A (500 μM) was made in ethanol, and of calmodulin (100 μM) in water. PKC, CaMK II, and calcineurin were pipetted directly into the bath.

Patch-clamp technique. Patch-clamp experiments were performed by the methods described previously [4]. Patch-clamp electrodes were pulled from hematocrit capillaries (Nichiden Rika Glass, Hyogo, Japan) and filled with the KCl solution, which was the same KCl solution ($10^{-7.5}$ M free Ca^{2+}) as that used for the bath. The electrode resistance ranged from 4 to 6 M Ω . Electric currents measured with a patch-clamp amplifier (EPC-8, HEKA Elektronik, Lambrecht, Germany), which was controlled by a Macintosh computer (PowerMacintosh 8500/150, Apple Computer, Cupertino, CA) equipped with interface (ITC-16, Instrutech, Elmont, NY) and control software (Pulse+ PulseFit, HEKA Elektronik), were stored on a DAT

Fig. 1. Representative current recordings of the inwardly rectifying K⁺ channel of OK proximal tubule cells in response to the application of ionomycin (1 μ M) with 10⁻⁶ M (A) and 10^{-7.5} M (B) of extracellular Ca²⁺. Summarized data of the effects of various concentrations in the presence of ionomycin (1 μ M) on channel activity in cell-attached patches (C). The channel recordings were made in a cell-attached patch with 50 mV pipette-holding potentials. Individual current levels of the K⁺ channels are indicated by short bars (left), and C. The summarized data are represented as channel activities normalized from control values. * Significant ($p < 0.001$) difference from control.

recorder (Model 5870, NF, Tokyo, Japan). The current records were played back and low-pass filtered (902LPF, Frequency Devices, Haverhill, MA) at 1 kHz and digitized at 1,000 samples/s with MacLab MK III (ADInstruments, New South Wales, Australia) and a Macintosh computer. The data obtained were analyzed by using the AXOGRAPH program system (Axon Instruments, Foster City, CA). Values for the potential were taken as the voltages of the pipette (V_p). Currents flowing from the cytoplasmic to the extracellular side were defined as outward (positive) and shown as upward deflections in the figures.

Channel activity was determined by the mean channel open probability (P_O), which was calculated as

$$NP_O = \sum_{n=1}^N (n \cdot t_n)$$

where N is the number of active channels in the patch and t_n ($t_1, t_2, \dots, t_N$) is the fractional open time at each current level. Normalized channel activity was calculated by the ratio $P_{O \text{ exp}}/P_{O \text{ cont}}$, where $P_{O \text{ cont}}$ and $P_{O \text{ exp}}$ are mean channel activities of control and experimental conditions, respectively. $P_{O \text{ cont}}$ was determined from 20 s data sampling period just before the addition of chemicals, and $P_{O \text{ exp}}$ was determined from 20 s data sampling period within the period of 30 to 90 s after the chemical had been added.

Statistics. Data are expressed as means $\pm$ SD. Where appropriate, one-way ANOVA is applied to assess the significance of difference for paired and unpaired samples.

RESULTS

First series of experiments

In the first series of experiments, we examined the effect of the Ca²⁺-PKC system on K⁺ channel activity in OKP cells by using ionomycin to clamp the intracellular Ca²⁺ concentration. Figure 1, A and B show representative single-channel recordings made in an increase in intracellular Ca²⁺ concentration on the inwardly rectifying K⁺ channel in cell-attached patches. Although an increase of the extracellular Ca²⁺ from 10^{-7.5} M to 10⁻⁶ M had no significant effect on channel activity, the addition of 1 μ M ionomycin to the bath in the presence of 10⁻⁶ M Ca²⁺ progressively decreased activity (Fig. 1A). This inhibitory effect of ionomycin on the K⁺ channel was fully reversible. Figure 1B shows that ionomycin had no significant effect on the channel activity when a 10^{-7.5} M Ca²⁺ concentration was used.

Figure 1C summarizes the results obtained from the ionomycin experiments in which cell Ca²⁺ was clamped at different levels, and it demonstrates a significant relationship between extracellular Ca²⁺ and K⁺ channel activity. K⁺ channel activity was reduced to $15.7 \pm 4.9\%$ of the control value ($n=5$) by raising intracellular Ca²⁺ to 10^{-6.5} M. The channel activity was further reduced to $13.4 \pm 8.3\%$ of the control value ($n=4$) by raising the extracellular Ca²⁺ concentration to 10⁻⁶ M bath Ca²⁺.

Figure 2 is a typical channel recording made in a cell-attached patch to show the effect of stimulation of PKC with 10⁻⁶ M Ca²⁺ on channel activity. The addition of 10 μ M PMA, phorbol ester known to stimulate PKC, decreased the activity. The removal of PMA gradually restored it, indicating the effect of PMA to

be reversible.

The changes in channel activity in response to the concentration of free Ca^{2+} from 10^{-8} to 10^{-5} M in inside-out patches are shown in Fig. 3. It was not influenced by the addition and removal of Ca^{2+} with 3 mM Mg-ATP in the bath.

To test whether PKC mediated inhibition of the channel activity, we examined the effect of exogenous PKC on the K^+ channels in inside-out patches in the presence of 10^{-6} or $10^{-7.5}$ M Ca^{2+} , respectively (Fig. 4). According to previous findings [14], we added 3 mM Mg-ATP and 10 μM PMA in inside-out patches to activate exogenous PKC. Although PMA had no significant effect on channel activity, the addition of exogenous PKC (1 U/ml) reversibly blocked the K^+ channel in the presence of 10^{-6} M Ca^{2+} (Fig. 4). In $10^{-7.5}$ M Ca^{2+} solution, the enzyme caused no significant change in channel activity (data not shown). These observations suggest that PKC-mediated phosphorylation is involved in the regulation of activity of the inwardly rectifying K^+ channel.

We observed that the addition of PKC did not reduce the channel activity ($99.6 \pm 1.3\%$; N.S., $n=4$) at a low concentration of Ca^{2+} ($10^{-7.5}$ M). With the increment of bath Ca^{2+} to $10^{-6.5}$ or 10^{-6} M, the addition of

PKC reduced it to $34.0 \pm 27.3\%$ ($p < 0.005$; $n=5$) and $32.5 \pm 18.4\%$ ($p < 0.001$; $n=5$), respectively. Furthermore, the PKC-induced inhibition of activity at the high concentration of bath Ca^{2+} was almost abolished by the pretreatment of 2 min with 5 μM GF 109203X ($96.7 \pm 5.1\%$; N.S.; $n=7$, data not shown), a competitive inhibitor of PKC. These data indicate that the activation of PKC produced the inhibition of inwardly rectifying K^+ channels of OKP cells.

As shown in Fig. 5, we examined the effect of GF 109203X (5 μM) on channel activity in cell-attached patches. The application of GF 109203X with or without 1 μM ionomycin in the 10^{-6} M Ca^{2+} solution had no significant effect on the channel activity. Compared with the experiment of ionomycin alone, shown in Fig. 1, this experiment showed that the inhibitory effect of ionomycin on the channel activity was abolished by pretreatment with the PKC inhibitor.

Second series of experiments

In the second series of experiments, we also studied the effect of the Ca^{2+} -calmodulin (CaM) system on the K^+ channel activity by using cyclosporin A, a selective inhibitor of calcineurin, and CaMK II. As shown in Fig. 6, the effect of CaMK II (300 pM) on this channel activity was examined in inside-out patches. CaMK II with 1 μM calmodulin and 10^{-6} M Ca^{2+} did not decrease channel activity; it dramatically increased the activity by $246.0 \pm 115.1\%$ ($p < 0.05$; $n=6$). This effect of CaMK II on the K^+ channel was completely different from the ROMK, as reported previously [11]. If CaMK II activates the K^+ channel in the whole cell condition, the application of GF 109203X with 1 μM ionomycin in the 10^{-6} M Ca^{2+} solution should produce the activation of this K^+ channel activity. However, an application of GF 109203X

Fig. 2. Changes in K^+ channel currents in response to PMA (10 μM) in a cell-attached patch. The channel recording was made with a 50 mV pipette-holding potential. The bath solution used was control NaCl solution.

Fig. 3. Effects of intracellular Ca^{2+} on the inwardly rectifying K^+ channel in the inside-out patch. **A:** The channel recordings were made with 50 mV pipette-holding potentials. The bath solution used was KCl solution containing 10^{-8} , 10^{-7} , 10^{-6} , or 10^{-5} M Ca^{2+} . The current level of the K^+ channel is indicated by short bars (left), and closed channel states are indicated by dotted lines and C. **B:** Summarized data of channel activity in the presence of various concentrations of Ca^{2+} in inside-out patches. Data are represented as channel activities normalized from control values.

Fig. 4. Representative current recordings of the inwardly rectifying K^+ channel of OK proximal tubule cells in response to the application of PKC (1 U/ml) with 10^{-6} M Ca^{2+} . The channel recordings were made in an inside-out patch with a 50 mV pipette-holding potential. Individual current levels of the K^+ channels are indicated by short bars (left) and C. Four parts of the recording indicated by short bars (A, B, C, and D) in the top trace are displayed in the expanded time course below.

Fig. 5. Changes in K^+ channel current in response to ionomycin (1 μ M) with GF 109203X (5 μ M). The channel recordings were made in cell-attached patches with a 50 mV pipette-holding potential. The bath solution used was NaCl solution containing 10^{-6} M Ca^{2+} .

with 1 μ M ionomycin in the 10^{-6} M Ca^{2+} solution produced no activation of this K^+ channel activity, as shown in Fig. 5. Thus in the next experiments, we ex-

Fig. 6. Representative current recordings of the inwardly rectifying K^+ channel of OK proximal tubule cells in response to the application of CaMK II (300 pM) with 10^{-6} M Ca^{2+} and 1 μ M calmodulin. The channel recordings were made in an inside-out patch with a 50 mV pipette-holding potential. Individual current levels of the K^+ channels are indicated by short bars (left) and C. Four parts of the recording indicated by short bars (A, B, C, and D) in the top trace are displayed in the expanded time course below.

amined the effect of the Ca^{2+} /calmodulin-dependent dephosphorylation process on this K^+ channel.

As shown in Fig. 7, we examined the effect of cyclosporin A (5 μ M) on the channel activity. Although the application of GF 109203X and cyclosporin A did not affect it, the subsequent application of ionomycin partially stimulated it within 1 min. Figure 8 shows the summarized data obtained from experiments similar to those shown in Figs. 1, 5, and 7. Channel activity maintained in the cell-attached patch with 10^{-6} M Ca^{2+} was significantly inhibited by ionomycin, but it was not affected by the application of GF 109203X alone ($94.6 \pm 1.3\%$; N.S.; $n=5$). An increase in activity was observed when GF 109203X and cyclosporin A were simultaneously added with ionomycin to the bath solution ($153.1 \pm 8.6\%$; $p < 0.001$; $n=4$). These results suggest that the channel was regulated by calcineurin (dephosphorylation process) or other Ca^{2+} -dependent phosphorylation processes.

Finally, the effect of calcineurin on channel activity was examined in inside-out patches (Fig. 9A). The ap-

Fig. 7. Changes in K^+ channel current in response to ionomycin ($1 \mu M$) with GF 109203X ($5 \mu M$) and cyclosporin A ($5 \mu M$), a selective inhibitor of calcineurin. The channel recordings were made in cell-attached patches with a $50 mV$ pipette-holding potential. The bath solution was a NaCl solution containing $10^{-6} M Ca^{2+}$.

Fig. 8. Summarized data of channel activity in response to ionomycin, ionomycin with GF 109203X, and ionomycin with GF 109203X and cyclosporin A in cell-attached patches. Ionomycin ($1 \mu M$, $n=5$), ionomycin with GF 109203X ($5 \mu M$, $n=5$), or ionomycin with GF 109203X and cyclosporin A ($5 \mu M$, $n=4$) were added to a bath solution containing $10^{-6} M Ca^{2+}$. Data are represented as channel activities normalized from control values. *Significant difference from control values at a level of $p < 0.001$.

Fig. 9. Effects of calcineurin ($2 U/ml$) on channel activity in the presence of $10^{-6} M Ca^{2+}$. **A:** Single-channel recordings in inside-out patches with a pipette-holding potential of $50 mV$. The current levels of the K^+ channel are indicated by a short bar (left) and closed channel states are indicated by the dotted lines and C. **B:** Summarized

data of the effects on channel activity of calcineurin in the presence of calmodulin, Ni^{2+} , and Ca^{2+} . Data are represented as normalized channel activities from control values. *Significant difference from control values at the level of $p < 0.001$.

plication of calcineurin with $10 \mu M Ni^{2+}$ and $1 \mu M$ calmodulin (calcineurin stimulator) in the $10^{-6} M Ca^{2+}$ solution decreased the activity by $74.9 \pm 3.6\%$ ($p < 0.001$; $n=5$) (Fig. 9B). The maximal effect of calcineurin on channel activity was observed within 1 min after the addition of calcineurin, and long-time exposure (for more than 3 min) of cell to calcineurin did not produce a more significant effect on the channel activity.

DISCUSSION

In the present study, we demonstrated that ATP-regulated K^+ channels in the OKP cells were affected by a Ca^{2+} -dependent phosphorylation/dephosphorylation process mediated by both Ca^{2+} -PKC and Ca^{2+} -CaM systems. Although the inhibitory effect of PKC on inwardly rectifying ATP-dependent K^+ channels in amphibian proximal tubule cells was already reported by

Mauerer *et al.* [13], CaM-dependent phosphorylation/dephosphorylation in the K^+ channel regulation process has not been reported in the proximal tubule cells.

The activity of the inwardly rectifying K^+ channels in OKP cells was reduced by the application of ionomycin with 10^{-6} M Ca^{2+} concentration in the bath solution (Fig. 1). However, the amplitude of single-channel current in pCa 6 with ionomycin was larger than in pCa 7.5 with ionomycin. The reason why single-channel current increased in increasing bath Ca^{2+} concentration with ionomycin is not clear, but it may be due to the intracellular pH changes, which produced the changes in single channel current in OKP cells, as reported previously [4], or to the different membrane potentials in the experiments of cell-attached patches at various Ca^{2+} concentrations. Nevertheless, the mean channel current calculated by single channel current and open probability should be decreased by increasing the cytosolic Ca^{2+} with ionomycin. Therefore the membrane potential in OKP cells would be depolarized at high cytosolic Ca^{2+} conditions such as pCa 6.0–6.5.

Several kinds of mechanisms for Ca^{2+} effects on channel activity have been considered, as follows: First, intracellular Ca^{2+} may inhibit the channel by direct binding, as indicated by Mauerer *et al.* [13]. Second, Ca^{2+} may inhibit through protein phosphorylation by PKC [13, 20] or CaMK II [11]. Third, calcineurin-mediated protein dephosphorylation may inactivate the channel [21].

Direct effect of Ca^{2+} on K^+ channel activity.

Several lines of evidence indicate that K^+ channels in the various nephron segments display direct Ca^{2+} -sensitivity. The large-conductance (>100 pS) K^+ channels observed in proximal tubules [22], thick ascending limb of Henle (TAL) [23], and cortical collecting tubule (CCT) [24, 25] were activated by intracellular Ca^{2+} . However, in these nephron segments intracellular Ca^{2+} had an inhibitory effect on intermediate- (30–100 pS) and small-conductance (<30 pS) K^+ channels; these effects were not direct [20, 26] except in ATP-sensitive K^+ channels in amphibian proximal tubule cells [13]. In the OKP cells, the activity of the inwardly rectifying K^+ channel did not depend on the Ca^{2+} concentration in inside-out patches (Fig. 3). This finding is consistent with observations in rat CCD that in excised patches without exogenous PKC, Ca^{2+} had no effect on channel activity [20, 26], suggesting that the Ca^{2+} -dependent inhibition of the K^+ channel is due to some Ca^{2+} -mediated process.

Effect of PKC on K^+ channel activity. We demonstrated that the K^+ channel activity in OKP

cells was inhibited by the addition of the phorbol ester, PMA, in cell-attached patches (Fig. 2) or directly by that of PKC with 10^{-6} M Ca^{2+} in inside-out patches (Fig. 4). Because the activation of PKC is elicited through Ca^{2+} -dependent signaling pathways, the inhibitory effect of intracellular Ca^{2+} on K^+ channel activity may occur via protein phosphorylation by PKC. Regarding the characteristics of PKC, it is reported that Ca^{2+} causes PKC to bind to the inner surface of the plasma membrane and that the activation of PKC requires diacylglycerol or membrane phosphatidylserine [27]. As shown in Fig. 5, GF109203X had no effect on this K^+ channel activity in cell-attached patches, suggesting that PKC has not been activated under the basal condition in OKP cells. Moreover, our study indicates that PKC molecules are not present in excised patch membranes because the addition only of Ca^{2+} did not inhibit the channel activity (Fig. 3). However, it is still undetermined whether PKC phosphorylates either the K^+ channel protein directly or, alternatively, a closely associated protein.

Channel inhibition by PKC has been reported for other K^+ channels [28, 29] including ATP-sensitive K^+ channels [30–32] in the kidney [20], and this effect of PKC is the opposite of that of PKA. The opposite regulation by PKA and PKC has been reported for the ATP-sensitive K^+ channels in amphibian proximal tubules [13]. These results suggest that Ca^{2+} -dependent PKC very likely plays a key role in the Ca^{2+} -mediated inhibition of the inwardly rectifying ATP-regulated K^+ channel. It has been demonstrated that PKC was able to inhibit Na^+ - K^+ ATPase transport activity by phosphorylating the α subunit of this ATPase [33]. Therefore PKC has an important physiological role in the pump-leak system across the basolateral membrane of proximal tubule cells.

Effect of CaMK II on K^+ channel activity. In the principal cells of the rat CCD, it has been demonstrated that raising the intracellular Ca^{2+} level reduced apical K^+ channel activity mediated by Ca^{2+} -dependent PKC and CaMKII [11, 13]. Mauerer *et al.* also suggested that Ca^{2+} inhibition of ATP-sensitive K^+ channels is Ca^{2+} /calmodulin-dependent protein kinase [13]. In the present study, however, the application of CaMK II dramatically increased this channel activity in inside-out patches, as shown in Fig. 6. This discrepancy may be due to the different characteristics between these K^+ channels, as indicated by Aguilar-Bryan *et al.*, who suggested the possibility of different pharmacological properties of ATP-sensitive K^+ channels with a tetrametric structure [7].

In the cell-attached patches in this study, the manipulation of raising the intracellular Ca^{2+} level did not

activate the K^+ channel, but instead inhibited it. Furthermore, under the inhibition of PKC with GF 109203X, the K^+ channel was not activated by raising intracellular Ca^{2+} in the presence of ionomycin (Fig. 5). In whole cell, therefore, the Ca^{2+} /calmodulin-dependent phosphorylation process does not work strongly to activate the channel or is inhibited by other unknown processes. However, we suspect that in cell-attached patches, as shown in Fig. 7, the increase in channel activity that occurred when the bath Ca^{2+} concentration was raised in the presence of GF 109203X and cyclosporin A might have been induced by Ca^{2+} /calmodulin-dependent protein kinase, such as CaMK II.

Effect of calcineurin on K^+ channel activity.

As indicated in Fig. 7, membrane-permeable calcineurin inhibitor cyclosporin A, along with GF 109203X, enhanced K^+ channel activity with application of ionomycin in cell-attached patches. It is possible that in cell-attached patches, the increase in activity obtained by raising bath Ca^{2+} in the presence of GF 109203X and cyclosporin A might have been due to a Ca^{2+} /calmodulin-dependent protein kinase, such as CaMK II. Therefore it is possible that the Ca^{2+} /calmodulin-dependent phosphorylation process might be offset by a Ca^{2+} /calmodulin-dependent dephosphorylation process. To confirm this possibility, we further examined the direct effects of calcineurin on channel activity in inside-out patches with 10^{-6} M Ca^{2+} solution. As we demonstrated in Fig. 9A, the channel activity maintained by 3 mM Mg-ATP was inhibited by calcineurin in the presence of Ni and calmodulin, both of which are known to activate calcineurin [21], suggesting that the Ca^{2+} /calmodulin-dependent phosphorylation process might be an offset by the Ca^{2+} /calmodulin-dependent dephosphorylation process in the cell-attached patches.

It was recently reported that cyclosporin A induced an increase in cytosolic Ca^{2+} [34]. In our study, however, cyclosporin A without ionomycin produced no changes in this channel activity, as we showed in Fig. 7. In our system, therefore, cyclosporin A induced no significant increase in cytosolic Ca^{2+} .

It is reported that calcineurin phosphatase activity in the proximal tubule is higher than in other nephron segments [35]. The physiological roles of calcineurin in the proximal tubule are still to be determined, but a few studies suggest that protein phosphatases also contribute to the regulation of Na^+ - K^+ ATPase activity in renal epithelia [36, 37]. However, these studies indicate that calcineurin activate Na^+ - K^+ ATPase. If calcineurin plays a physiological role in the pump-leak coupling of K^+ across the basolateral membrane

of proximal tubule cells, it should not inhibit K^+ channel activity, but instead activate the K^+ channel. Not only calcineurin, but also PKA has been shown to have contradictory effects on the pump-leak system in the basolateral membrane of proximal tubule cells; i.e., PKA activates the ATP-regulated K^+ channel [4–6] and inhibits Na^+ - K^+ ATPase [38, 39]. Further studies are needed to explain this discrepancy.

From our present studies on OKP cells, we conclude that Ca^{2+} -dependent PKC plays a key role in the Ca^{2+} -mediated inhibition of the inwardly rectifying ATP-regulated K^+ channel and that the Ca^{2+} /calmodulin-dependent phosphorylation process is offset by a Ca^{2+} /calmodulin-dependent dephosphorylation process.

In regard to the physiological significance underlying the inhibitory effect of an increase in cytosolic Ca^{2+} on this K^+ channel, our results suggest that the dramatic increase in cytosolic Ca^{2+} induced by ischemia [40–42] may protect K^+ loss or cell damage from the proximal tubular cells. Furthermore, the lack of an inhibitory effect of low Ca^{2+} concentrations (less than 10^{-7} M) on the K^+ channel is believed to play an important role in maintaining membrane potential under physiological conditions, since the intracellular Ca^{2+} concentration is less than 10^{-7} M in the proximal tubule cells [19]. Further study will be necessary to clarify the regulatory mechanism governing this K^+ channel.

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