Regulation of Inwardly Rectifying K⁺ Channel in Cultured Opossum Proximal Tubule Cells by Protein Phosphatases 1 and 2A

Manabu KUBOKAWA, Kazuyoshi NAKAMURA, Junko HIRANO, Yoshichika YOSHIOKA, Shigeyuki NAKAYA, Yoshiaki MORI*, and Takahiro KUBOTA*

Department of Physiology II, School of Medicine, Iwate Medical University, Morioka, 020–8505 Japan; and

*Department of Physiology, Osaka Medical College, Takatsuki, 569–8686 Japan

Abstract: The inwardly rectifying ATP-regulated K⁺ channel with an inward conductance of about 90 pS in the surface membrane of cultured opossum kidney proximal tubule (OKP) cells is activated at least in part by protein kinase A (PKA). In this study, we examined the effects of protein serine/threonine phosphatase types 1 (PP-1) and 2A (PP-2A) on activity of the K⁺ channel using the patch-clamp technique. In cell-attached patches, channel activity was enhanced by the application of okadaic acid (OA, 1 μM), a membrane-permeable inhibitor of PP-1 and PP-2A, to the bath solution. This enhancement was abolished by the pretreatment of cells with KT5720 (200 nM), a specific inhibitor of PKA. In inside-out patches, channel activity which could be maintained in the presence of ATP (3 mM) in the bath solution was also increased by the addi-

tion of OA (1 μM), and the OA-induced increase in channel activity was partially prevented in the presence of KT5720 (200 nM). Direct application of either PP-1 (1 U/ml) or PP-2A (1 U/ml) to the cytoplasmic surface of the patch membrane inhibited channel activity maintained by ATP (3 mM) in inside-out patches. Moreover, channel activity stimulated by PKA (20 nM) in the presence of ATP (3 mM) was also inhibited by the application of either PP-1 (1 U/ml) or PP-2A (1 U/ml). These results indicate that the OA-sensitive protein phosphatase is involved in the regulation of channel activity, and suggest that both PP-1 and PP-2A are candidates responsible for the inhibition of channel activity through dephosphorylation of the PKA-mediated protein phosphorylation. [Japanese Journal of Physiology, 50, 249–256, 2000]

Key words: kidney, patch-clamp, phosphorylation, dephosphorylation, okadaic acid.

Renal potassium channels along the nephron play important roles in several functions of the kidney, such as formation of the membrane potential of tubule cells, which serves as a driving force for ion transport processes in tubular epithelia, or K⁺ secretion through the channels in the distal nephron segment [1–3]. The protein kinase-mediated phosphorylation of serine/threonine residue is one of the important processes in mediating channel activity. It has been demonstrated in the basolateral membrane of *Ambystoma* proximal

tubule cells that the inwardly rectifying K⁺ channel with inward conductance of about 24.5 pS is activated by cAMP-dependent protein kinase (PKA) and inhibited by protein kinase C (PKC) [4]. We have also demonstrated in cultured opossum proximal tubule (OKP) cells that the inwardly rectifying K⁺ channel with an inward conductance of about 90 pS is activated by PKA [5] and inhibited by PKC [6]. More recently, we showed the involvement of guanosine 3',5'-cyclic monophosphate (cGMP)-dependent protein ki-

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Correspondence should be addressed to: Manabu Kubokawa, Department of Physiology II, School of Medicine, Iwate Medical University, 19–1, Uchimaru, Morioka, 020–8505 Japan. Tel: +81–19–651–5111, Fax: +81–19–654–8341, E-mail: mkubokaw@iwate-med.ac.jp

nase (PKG) in activation of the K^+ channel in OKP cells [7]. In the principal cell of rat cortical collecting duct (CCD), the basolateral K^+ channel is activated by PKC [8] and PKG [9], and the apical K^+ channel, which is responsible for K^+ secretion, is activated by PKA and inhibited by PKC [10] and Ca^{2+} /calmodulin-dependent protein kinase II [11]. Apical K^+ channels in the thick ascending limb of the rat were reported to be activated by PKA [12].

In addition to protein kinases, protein phosphatases are also important in the regulation of channel activity via dephosphorylation processes. We have previously demonstrated in principal cells of the rat CCD that protein serine/threonine phosphatase type 2A directly inhibits activity of the apical K^+ channel, which is stimulated by ATP and PKA in inside-out patches, suggesting that protein phosphatase-mediated dephosphorylation plays a key role in the regulation of channel activity [13]. However, in the proximal tubule cells, little is known about the phosphatase-mediated process in regulating channel activity.

In this study, we examined the involvement of protein serine/threonine phosphatase types 1 (PP-1) and 2A (PP-2A) in the regulation of K^+ channel activity in OKP cells. We demonstrate that both PP-1 and PP-2A are able to inhibit channel activity via dephosphorylation processes of PKA-mediated phosphorylation of the channel or associated protein.

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 described previously [14]. The cells were isolated from confluent monolayers at passages 18–48 with trypsin and incubated on collagen-coated cover glasses (Iwaki, Tokyo, Japan) with a culture medium for 4–8 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). The KCl solution used for the bath in inside-out patches contained (in mM) 115 KCl, 3 $MgCl_2$, 30 KOH, 10 ethyleneglycol-bis(β -aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), and 5 HEPES with $10^{-7.5}$ M free Ca^{2+} . The free Ca^{2+} concentration was adjusted by adding an adequate amount of $CaCl_2$ according to the computer program of Oiki and Okada [15], which was made using the absolute values of stability constant of EGTA for the

binding of Ca^{2+} , Mg^{2+} , and H^+ . The solutions with ATP were made by adding Na_2ATP (Nacalai Tesque, Kyoto, Japan). The pH of these solutions was adjusted to 7.3 with a minute amount of HCl, NaOH or KOH at 24°C.

Chemicals. Protein phosphatase types 1 (PP-1) and 2A (PP-2A) were purchased from Upstate Biotechnology Incorporated (Lake Placid, NY, USA). Catalytic subunit of PKA and KT5720, a specific inhibitor of PKA [16], were purchased from Calbiochem (La Jolla, CA, USA). Okadaic acid (OA), an inhibitor of PP-1 and PP-2A [17, 18], was obtained from Wako (Osaka, Japan).

Patch-clamp technique. Patch-clamp experiments were performed by methods described previously [7]. Patch-clamp electrodes were pulled from hematocrit capillaries (Nichiden Rika, Hyogo, Japan) and filled with KCl solution, the same KCl solution used for the bath solution in inside-out patches. The electrode resistance ranged between 3 and 5 M Ω . Electric currents measured with a patch-clamp amplifier (AXOPATCH 200A, Axon, Foster City, CA, USA) were stored on a DAT recorder (Model 5870, NF, Tokyo, Japan). The current records were then low-pass filtered (902LPF, Frequency Devices, Haverhill, MA, USA) at 0.3–1 kHz, and analyzed using the AXOGRAPH program system (Axon, Foster City, CA, USA). Values for the potential represent the voltages of the pipette (V_p). Currents flowing from the cytoplasmic to the extracellular side were defined as outward and shown as upward deflections in the figures.

Channel activity was determined by 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 membrane, and t_n ($t_1, t_2, \dots, t_N$) is the fractional time spent at each current level. Normalized channel activity was calculated by the ratio $NP_{O \text{ exp}}/NP_{O \text{ cont}}$, where $NP_{O \text{ cont}}$ and $NP_{O \text{ exp}}$ are the mean channel activities of control and experimental conditions, respectively. $NP_{O \text{ cont}}$ was determined from a 20-s data sampling period just before adding chemicals, and $NP_{O \text{ exp}}$ was determined from a 10-s data sampling after adding the chemicals (see figure legends for the time of the sampling period in detail).

Statistics. Data are expressed as means $\pm$ SE. Statistical significance was determined using Student's t -test or ANOVA in conjunction with the Bonferroni t -test. A p value less than 0.05 was considered to be significant.

Fig. 1. Effects of okadaic acid (OA) on activities of the inwardly rectifying K⁺ channel of OKP cells with or without KT5720 in cell-attached patches.

A: A representative current recording of the K⁺ channel in response to the application of okadaic acid (OA, 1 μM). The channel recording was made in a cell-attached patch with a 0 mV pipette holding potential. Short bars (left) indicate individual current levels of K⁺ channels, and dotted lines and C indicate closed channel states. **B:** K⁺ channel currents in response to KT5720 (200 nM) following the addition of OA (1 μM). The channel recordings were made with a 0 mV pipette holding potential. Individual current levels of K⁺ channels are indicated by short bars (left) and closed channel

states are indicated by dotted lines and C. Four parts of the recording indicated by short bars (1–4) in the top trace are displayed in an expanded time course below. **C:** Summarized data of effects of OA (1 μM), KT5720 (200 nM), and OA (1 μM) in the presence of KT5720 (200 nM) on channel activity. Data of the OA effect on channel activity in the absence or presence of KT5720 were obtained 30 to 60 s after adding the chemical, and that of the effect of KT5720 were obtained 30 to 90 s after adding it. Individual data are represented as normalized channel activities from control values. *Significant difference from control values on paired samples. Numbers in parentheses above the bars indicate the number of experiments.

RESULTS

An inwardly rectifying K⁺ channel with inward conductance of about 90 pS was observed in more than 80% of the patch-membrane in OKP cells [5, 14]. This channel was easily distinguished from other ion channels, such as large conductance maxi-K⁺ channels or small conductance channels, present in the surface membrane of OKP cells by voltage steps of the pipette potential. It is also of interest that the presence of Ca²⁺ in the pipette solution suppresses the inward conductance of inwardly rectifying K⁺ channels [19]. However, the very low concentration of Ca²⁺ (pCa 7.5) in the pipette adopted in this study induced no appreciable change in the inward channel conductance.

Figure 1 shows a representative current recording of the inwardly rectifying K⁺ channel in response to the application of OA (1 μM) into the bath solution in a cell-attached patch. In the control condition, the patch membrane contained two active channels and the application of OA activated two more channels. Although the OA effect on channel activity was sometimes irreversible as shown in Fig. 1A, this result indicates that OA activates the inwardly rectifying K⁺ channel. Since OA is an inhibitor of PP-1 and PP-2A at a concentration of around 1 μM [17], it is suggested that the OA-sensitive protein phosphatase, PP-1 or PP-

2A, is involved in modulation of the K⁺ channel activity.

In a previous paper, we have demonstrated that PKA-mediated phosphorylation is important in the stimulation of channel activity [5]. Thus, in the next step, we examined the effect of OA on channel activity in the presence of KT5720, a specific inhibitor of PKA [16], in cell-attached patches to examine whether OA-induced stimulation of channel activity is predominantly produced by PKA-mediated phosphorylation. As shown in Fig. 1B, KT5720 (200 nM) gradually inhibited channel activity, suggesting that channel activity maintained in cell-attached patches results from at least, in part, PKA-mediated phosphorylation, and the following application of OA induced an only slight increase in channel activity. Namely, the OA effect on channel activity was abolished when a PKA inhibitor was present.

Summarized data similar to those represented in Fig. 1A and B are shown in Fig. 1C. The application of OA significantly stimulated channel activity in cell-attached patches, and KT5720 significantly inhibited channel activity. Furthermore, the OA-induced channel activation was significantly abolished when channel activity was inhibited by KT5720. Since inhibition of the phosphatase blocks dephosphorylation of the corresponding phosphorylation site, these results sug-

Fig. 2. Effects of OA on activities of the K^+ channel with or without KT5720 in inside-out patches. **A:** K^+ channel currents in response to OA ($1 \mu\text{M}$). The solutions in pipette and bath were symmetrical KCl solutions without the presence of 3 mM ATP in

the bath solution. The pipette holding potential was $+50 \text{ mV}$. Short bars (left) indicate individual current levels of K^+ channels, and dotted lines and C indicate closed channel states. **B:** Changes of channel activity in response to KT5720 (200 nM) following the addition of OA ($1 \mu\text{M}$) in an inside-out patch. The pipette and bath solutions were KCl solutions, and the bath solution contained 3 mM ATP. The channel recordings were made with a $+50 \text{ mV}$ pipette holding potential. Short bars (left) indicate individual current levels of K^+ channels, and dotted lines and C indicate closed channel states. Four parts of the recording indicated by short bars (1–4) in the top trace are displayed in an expanded time course below. **C:** Summarized data of effects of OA ($1 \mu\text{M}$), KT5720 (200 nM), and OA ($1 \mu\text{M}$) in the presence of KT5720 (200 nM) on channel activity. Data of the effect of KT5720 or OA in the absence or presence of KT5720 on channel activity were obtained 20 to 60 s after adding the chemicals and are represented as normalized channel activities from control values. * Significant difference from control values on paired samples. Numbers in parentheses above the bars indicate the number of experiments.

gest that OA leads PKA-mediated phosphorylation dominantly, resulting in the stimulation of channel activity.

In inside-out patches, ATP was required to maintain channel activity in the bath solution, and the ATP-dependent channel activity reached near maximum at the ATP concentration of 3 mM [5]. Thus, the following experiments in inside-out patches were carried out in the presence of 3 mM ATP as the control condition. Figure 2A shows a recording of channel activity in response to OA in an inside-out patch. The addition of OA ($1 \mu\text{M}$) markedly stimulated channel activity, suggesting the presence of membrane-bound OA-sensitive phosphatases.

Furthermore, we examined the OA effect on channel activity in the presence of KT5720 (200 nM) in inside-out patches. As shown in Fig. 2B, KT5720 inhibited channel activity, although it was not complete, suggesting that channel activity maintained by ATP in inside-patches was induced, at least in part, by membrane-bound PKA. The following addition of OA ($1 \mu\text{M}$) moderately enhanced channel activity, but it was not so remarkable. These results suggest that OA alone is not enough to enhance channel activity by in-

hibiting the OA-sensitive phosphatase, but requires PKA-mediated phosphorylation for marked channel activation.

The data obtained from the experiments similar to those represented in Fig. 2A and B are summarized in Fig. 2C. OA ($1 \mu\text{M}$) significantly enhances channel activity while KT5720 (200 nM) significantly inhibits it. Moreover, OA-induced channel activation is significantly prevented in the presence of KT5720, suggesting that significant channel activation induced by OA requires PKA-mediated phosphorylation.

The effects of exogenous PP-1 and PP-2A on channel activity were examined in inside-out patches. Changes in channel activity in response to direct application of PP-1 (1 U/ml) and PP-2A (1 U/ml) are shown in Fig. 3A and B, respectively. Both PP-1 and PP-2A reversibly inhibited channel activity. The summarized data of direct effects of PP-1 and PP-2A on channel activity in inside-out patches are represented in Fig. 3C. Either PP-1 or PP-2A induced significant inhibition of channel activity, suggesting that these phosphatases are effective for dephosphorylation of the channel or associated protein.

Finally, we examined the effects of PP-1 and PP-2A

Fig. 3. Effects of direct application of PP-1 or PP-2A on channel activity in inside-out patches. **A:** Changes in channel activity in response to the application of PP-1 (1 U/ml) to an inside-out patch. The channel recording was made with a +50 mV pipette holding potential, and the solutions in pipette and bath were symmetrical KCl solutions without the presence of 3 mM ATP in the bath solution. Short bars (left) indicate individual current levels of K⁺ channels, and dotted lines and C indicate closed channel states. Three parts of the recording indicated by short bars (1–3) in the top trace are displayed in an expanded time course below. **B:** Changes in channel activity in response to the application of PP-2A (1 U/ml) in an inside-out patch. Experimental conditions and explanation of this figure are the same as those described above (A). **C:** Summarized data of effects of PP-1 (1 U/ml) or PP-2A (1 U/ml) on channel activity. Data were obtained 30 to 60 s after adding PP-1 or PP-2A, and are represented as normalized channel activities from control values. * Significant difference from control values on paired samples. Numbers in parentheses above the bars indicate the number of experiments.

on channel activity in the presence of PKA (20 nM) in inside-out patches. As shown in Fig. 4A, PKA enhanced channel activity, and the inhibitory effects of PP-1 on channel activity were observed even when PKA (20 nM) was present. PP-2A (1 U/ml) also inhibited channel activity even in the presence of PKA (20 nM) (data not shown). The direct effects of PP-1 and PP-2A on channel activity in the presence of PKA are summarized in Fig. 4B. PKA significantly stimulated channel activity, and both PP-1 and PP-2A significantly inhibited PKA-mediated stimulation of channel activity. These data indicate that OA-sensitive PP-1 and PP-2A are both effective for the dephosphorylation process of PKA-mediated phosphorylation.

DISCUSSION

Phosphorylation and dephosphorylation processes are important in the regulation of several kinds of ion channels. In the renal K⁺ channels, the role of phos-

phorylation mediated by protein kinases in the regulation of channel activity has extensively been examined in the basolateral K⁺ channel of proximal tubule cells [4] and the basolateral and apical K⁺ channels in principal cells of CCD [8–11]. As for the dephosphorylation process, however, only a few papers have suggested that channel activities enhanced by PKA-mediated phosphorylation are inhibited by phosphatase-mediated dephosphorylation in the apical K⁺ channel of rat CCD [13] or cloned renal K⁺ channels (ROMK) expressed in *Xenopus* oocyte [20]. In the present study, we demonstrated the involvement of OA-sensitive protein phosphatases in the regulation of the inwardly rectifying K⁺ channel in OKP cells.

It has been reported that the surface membrane of OKP cells largely corresponds to the basolateral membrane of proximal tubule cells [14]. One of the significant features of the inwardly rectifying K⁺ channel present in the surface membrane of OKP cells is that ATP (1–3 mM) is required to maintain channel

Fig. 4. Changes in channel activity in response to PKA following the addition of PP-1 or PP-2A in inside-out patches.

A: The channel recording was made with a +50 mV pipette holding potential. The solutions in pipette and bath were symmetrical KCl solutions without the presence of 3 mM ATP in the bath solution. Short bars (left) indicate individual current levels of K⁺ channels, and dotted lines and C indicate closed channel states. Four parts of the recording indicated by short bars (1–4) in the top trace are displayed in an expanded time course below. **B:** Summarized data of channel activity in response to PKA (20 nM) alone, and to PKA (20 nM) with PP-1 (1 U/ml) or PP-2A (1 U/ml). Data of the effect of PKA (20 nM) on channel activity were obtained 30 to 90 s after adding the kinase, and that of the effect following the addition of PP-1 or PP-2A were obtained 30 to 90 s after adding the individual phosphatases. Individual data are represented as normalized channel activities from control values. * Significant difference from control values on paired samples. Numbers in parentheses above the bars indicate the number of experiments.

activity in inside-out patches [5]. Furthermore, we suggested in a previous paper that the ATP-dependent channel activity results from phosphorylation by membrane-bound PKA since PKA inhibitor diminished the activity despite the absence of exogenous PKA in inside-out patches [5]. In this study, we showed that OA, an inhibitor of PP-1 and PP-2A, enhanced the K⁺ channel activity in both cell-attached (Fig. 1A and C) and inside-out patches (Fig. 2A and C), suggesting that PP-1 and/or PP-2A are involved in regulating channel activity via protein dephosphorylation. A similar result, that OA enhances channel activity, has also been demonstrated in other ion channels [21–23]. Moreover, the result that OA enhanced channel activity in inside-out patches (Fig. 2A and C), and that this enhancement was abolished by a PKA inhibitor, KT5720, indicates the presence of membrane-bound PP-1 and/or PP-2A as well as membrane-bound PKA. Thus, it is conceivable that channel activation induced by inhibition of these phosphatases results predominantly from PKA-mediated phosphorylation. This notion is supported by experiments in which inhibition of PKA abolished OA-induced channel activation (Figs. 1 and 2). Taken together, these experiments using a PKA inhibitor and OA suggest that channel activation induced by PKA-mediated phosphorylation can be inhibited by PP-1- and/or PP-2A-mediated dephosphorylation.

The direct application of either PP-1 or PP-2A to the cytoplasmic surface of patch membrane inhibits

channel activity in inside-out patches (Fig. 3), indicating that both PP-1 and PP-2A are able to dephosphorylate the channel or associated protein. In the final experiments, we examined the effects of PP-1 and PP-2A on channel activity in the presence of exogenous PKA in inside-out patches. As shown in Fig. 4B, both PP-1 and PP-2A inhibited channel activity even when the channel was activated by exogenous PKA. These results provide direct evidence that PKA-mediated phosphorylation can be dephosphorylated by either PP-1 or PP-2A. Thus, both PP-1 and PP-2A are candidates responsible for the dephosphorylation process of PKA-mediated phosphorylation, although it is still unclear which of the phosphatases is influential in intact OKP cells. It is also suggested that channel activity is determined, at least in part, by the balance of the phosphorylation and dephosphorylation, and that the condition we adopted in the final experiments (concentrations of PKA and the phosphatases were 20 nM and 1 U/ml, respectively) resulted in the phosphatase-mediated dephosphorylation dominating over the PKA-mediated phosphorylation.

In the apical K⁺ channel in the principal cell of rat CCD, we demonstrated that PKA-induced channel activation was directly inhibited by PP-2A but not by PP-1 [13], suggested that PP-1 is not responsible for dephosphorylation of the PKA-mediated phosphorylation. It has also been suggested that PP-2A, but not PP-1, is involved in the dephosphorylation processes of PKA-mediated phosphorylation in Ca²⁺-activated

K⁺ channels [24], epithelial Cl⁻ channels [25], and the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel [26]. These data are inconsistent with the present data showing the involvement of both PP-1 and PP-2A in the dephosphorylation of PKA-mediated phosphorylation. Although this discrepancy would make it complex to understand the specificity of protein kinase and phosphatase in reversible phosphorylation processes, our data suggest that the PKA-mediated phosphorylation site is not identical but variable. Indeed, it has recently been shown that PKA-mediated phosphorylation is dephosphorylated by PP-1 in the NMDA receptor [27].

The present paper provided evidence that both PP-1 and PP-2A inhibited K⁺ channel activity through the dephosphorylation of PKA-mediated phosphorylation in OKP cells. However, the involvement of other protein serine/threonine phosphatases, such as types 2B and 2C, in the dephosphorylation process is still unknown. Furthermore, we demonstrated in previous papers that, besides the PKA-mediated process, cGMP-dependent [7] and PKC-mediated phosphorylation [6] are involved in the regulation of K⁺ channel activity. Also in these phosphorylation processes, reversible dephosphorylation by some phosphatases would be indispensable. Additional experiments will be necessary to more precisely clarify the mechanism for regulation of channel activity by phosphorylation and dephosphorylation processes.

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