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European Journal of Pharmacology 408 (2000) 107–116

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Phosphorylation of protein phosphatase-1 inhibitors, inhibitor-1 and DARPP-32, in renal medulla

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Received 20 July 2000; received in revised form 2 October 2000; accepted 6 October 2000

Abstract

Inhibitor-1 and DARPP-32 (dopamine and cAMP-regulated phosphoprotein, Mr 32 kDa) are each phosphorylated by cAMP-dependent protein kinase, resulting in their conversion to potent inhibitors of protein phosphatase-1. Protein phosphatase-1 is involved in the regulation of Na^+ reabsorption from renal tubule by modulating the activity of Na^+,K^+-ATPase. In this study, we have investigated the regulation of inhibitor-1 and DARPP-32 phosphorylation in slices of renal medulla. Activation of cAMP-dependent protein kinase by forskolin and 8-bromo-cAMP increased the level of phosphorylated inhibitor-1. Okadaic acid (1 μM), used to inhibit protein phosphatase-2A, increased the level of phosphorylated inhibitor-1, but cyclosporin A had no effect. DARPP-32, like inhibitor-1, was phosphorylated by cAMP-dependent protein kinase and dephosphorylated only by protein phosphatase-2A. These data demonstrate that the phosphorylation of inhibitor-1 and DARPP-32 is regulated by the balance of phosphorylation by cAMP-dependent protein kinase and dephosphorylation by protein phosphatase-2A in renal medulla. Furthermore, the phosphorylation step is regulated by pharmacological stimuli such as activation of β_1-adrenoceptors and dopamine D1 receptors.

Keywords: cAMP-dependent protein kinase; Adrenoceptor; Dopamine; Loop of Henle; (Mouse)

1. Introduction

The regulation of Na^+ reabsorption from renal tubule is critical in the homeostasis of Na^+ and water balance. Na^+,K^+-ATPase plays a central role in the Na^+ reabsorption, and its activity is regulated by various hormones such as catecholamines. Dopamine has been shown to inhibit Na^+,K^+-ATPase activity by activating both dopamine D1- and D2-type receptors in proximal tubule and by activating dopamine D1-type receptors in the medullary thick ascending limb of Henle and collecting duct (Aperia, 2000). Activation of dopamine D1-type receptors, which is an essential component of dopamine signaling, stimulates cAMP production and cAMP-dependent protein kinase activity. The activation of cAMP-dependent protein kinase increases the phosphorylation of its substrates, leading to the inhibition of Na^+,K^+-ATPase activity. However, the downstream signaling cascade of cAMP-dependent protein kinase in renal tubule is not well characterized. It has been reported that the α1 isoform of Na^+,K^+-ATPase is phosphorylated at Ser^{943} (rat sequence) by cAMP-dependent protein kinase in vitro and in cell lines overexpressing the α1 isoform (Fisone et al., 1994; Begiun et al., 1996; Cheng et al., 1997). This phosphorylation is associated with the inhibition of enzyme activity (Fisone et al., 1994). There are also reports using intact tissues showing that activation of cAMP-dependent protein kinase does not directly phosphorylate Na^+,K^+-ATPase, but inhibits Na^+,K^+-ATPase activity by phosphorylating other substrates that indirectly inhibit Na^+,K^+-ATPase activity (Feschenko and Sweadner, 1995; Nishi et al., 1999a). In these cases, DARPP-32 (dopamine and cAMP-regulated phosphoprotein, Mr 32 kDa) and/or inhibitor-1 seem to mediate the effects of cAMP-dependent protein kinase, since the inhibition of protein phosphatase-1 activity was associated with the reduction of Na^+,K^+-ATPase activity in medullary thick ascending limb of Henle (Aperia et al., 1991; Li et al., 1995) and, furthermore, the inhibitory effect of dopamine on Na^+,K^+-ATPase activity was lost in

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00767-6

neostriatal neurons from DARPP-32 mutant mice (Fienberg et al., 1998; Nishi et al., 1999b).

DARPP-32 is a cytosolic protein that is enriched in medium-sized spiny neurons in neostriatum (for review, see Greengard et al., 1999). Inhibitor-1 is also a cytosolic protein that is ubiquitously expressed in almost all tissues (Hemmings et al., 1992). Inhibitor-1 (at Thr^{35}) and DARPP-32 (at Thr^{34}) are each phosphorylated by cAMP-dependent protein kinase, resulting in their conversion to potent inhibitors of protein phosphatase-1. Phosphorylated forms of inhibitor-1 and DARPP-32 are dephosphorylated by protein phosphatase-2A and calcineurin in vitro (Hemmings et al., 1984a; King et al., 1984). It has been shown that inhibitor-1 and DARPP-32 are highly expressed in a nephron segment of medullary thick ascending limb of Henle (Meister et al., 1989). The inhibition of protein phosphatase-1 activity by phospho-Thr^{34} DARPP-32 peptide and calyculin A resulted in the inhibition of Na^+,K^+-ATPase activity (Aperia et al., 1991; Li et al., 1995), indicating that protein phosphatase-1 plays a critical role in the regulation of Na^+ reabsorption by modulating the activity of Na^+,K^+-ATPase. Thus, inhibitor-1 and DARPP-32 seem to be physiological inhibitors of protein phosphatase-1 in renal tubule, but there is no direct evidence that phosphorylation of inhibitor-1 and/or DARPP-32 is regulated in renal tubule. In this study, we investigated the regulation of inhibitor-1 and DARPP-32 phosphorylation in slices of mouse renal medulla. This is the first report demonstrating inhibitor-1/DARPP-32 phosphorylation in renal tissue by reagents, activating cAMP-dependent protein kinase or inhibiting protein phosphatase-2A, and by pharmacological stimuli.

2. Material and methods

2.1. Preparation and incubation of slices

Male C57BL/6 mice (6–8 weeks old) were anesthetized with diethyl ether and sodium pentobarbital (50 mg/kg body weight, i.p.), and the kidneys rapidly removed and placed in ice-cold, oxygenated Krebs–HCO_3^- buffer (124 mM NaCl, 4 mM KCl, 26 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1.25 mM KH_2PO_4, 1.5 mM $MgSO_4$ and 10 mM D-glucose, pH 7.4). Coronal slices (350 μm) were prepared using a vibratome. Renal medulla was dissected from the slices in ice-cold Krebs–HCO_3^- buffer. Each slice was placed in a polypropylene incubation tube with 2 ml of fresh Krebs–HCO_3^- buffer containing adenosine deaminase (10 μg/ml) and sodium butyrate (1 mM). The slices were pre-incubated at 30°C under constant oxygenation with 95% O_2/5% CO_2 for 60 min. The buffer was replaced with fresh Krebs–HCO_3^- buffer after 30 min of pre-incubation. Slices were treated with drugs as specified in each experiment. Drugs were obtained from the following sources: (±)-6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide (SKF81297), (±)-2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1*H*-imidazol-2-yl]phenoxy]-propyl]amino]ethoxy]-benzamide methanesulfonate (CGP20712A), (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride (ICI118551), 2-[(2-cyclopropylphenoxy)methyl]-4,5-dihydro-1*H*-imidazole hydrochloride (cirazolin), and 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine (UK14304) from Research Biochemicals International (Natik, MA); forskolin, cyclosporin A, okadaic acid and calyculin A from Alexis Biochemicals (San Diego, CA); 8-bromo-cAMP, 8-bromo-cGMP, isoproterenol and 3-[(4,5-dihydro-1*H*-imidazol-2-yl)methyl]-6-(1,1-dimethylethyl)-2,4-dimethylphenol hydrochloride (oxymetazoline) from Sigma (St. Louis, MO); Calcimycin (A23187) from Calbiochem (San Diego, CA). After the drug treatment, slices were transferred to Eppendorf tubes, frozen on dry ice, and stored at −80°C until assayed.

2.2. Immunoblotting

Frozen tissue samples were sonicated in boiling 1% sodium dodecyl sulfate (SDS) and boiled for an additional 10 min. Small aliquots of the homogenate were retained for protein determination by the bicinchoninic acid (BCA) protein assay method (Pierce, Rockford, IL) using bovine serum albumin as a standard. Equal amounts of protein

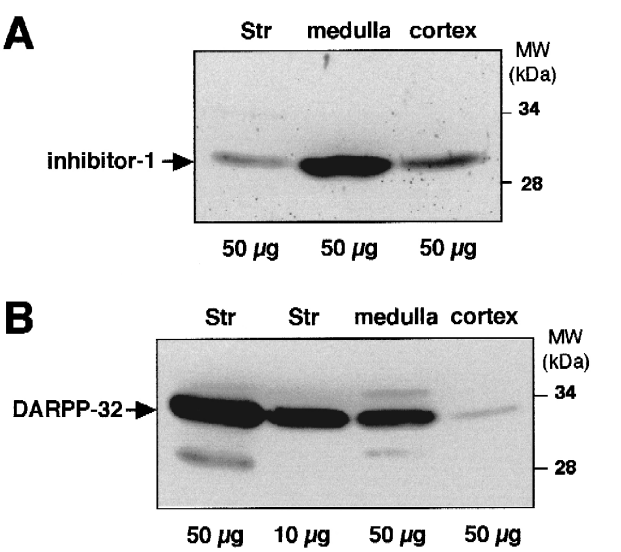

Fig. 1. Concentrations of inhibitor-1 and DARPP-32 in striatal and renal tissues. Homogenates from mouse striatum (Str), renal medulla and renal cortex (50 or 10 μg) were loaded on 12% SDS-PAGE. (A) Inhibitor-1 was detected at a molecular mass of ~29 kDa using a polyclonal antibody (G185) against inhibitor-1. (B) DARPP-32 was detected at a molecular mass of ~32 kDa using a monoclonal antibody (C24-5a) against DARPP-32.

(120–200 μg) were loaded onto 12% acrylamide gels, the proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose membranes (0.2 μm) (Schleicher and Schuell, Keene, NH) as described (Towbin et al., 1979). The membranes were immunoblotted using a monoclonal antibody (mAb-23; 1:750 dilution) (Snyder et al., 1992), which is a phosphorylation state-specific antibody raised against a DARPP-32 peptide containing phospho-Thr[34], the site phosphorylated by cAMP-dependent protein kinase. The antibody recognized both phospho-Thr[34] DARPP-32 and phospho-Thr[35] inhibitor-1 due to the high degree of sequence homology at the cAMP-dependent protein kinase sites of two proteins. A monoclonal antibody (C24-5a; 1:7500 dilution) generated against DARPP-32 (Hemmings and Greengard, 1986) and a polyclonal antibody generated against inhibitor-1 (G185; 1:2000 dilution), which are not phosphorylation state-specific, were used to estimate the total amounts of DARPP-32 and inhibitor-1. None of the experimental manipulations used in the present study altered the total amount of DARPP-32 or inhibitor-1.

Antibody binding was revealed by incubation with goat anti-mouse horseradish peroxidase-linked immunoglobulin G (IgG) (1:2000–4000 dilution) or goat anti-rabbit horseradish peroxidase-linked IgG (1:5000 dilution) (Pierce) and the Enhanced Chemiluminescence (ECL) immunoblotting detection system (Amersham, Arlington Heights, IL). Chemiluminescence was detected by autoradiography using Kodak autoradiography film, and phospho-inhibitor-1 bands were quantified by densitometry using National Institutes of Health (NIH) Image 1.61 software. Since the linear range for quantitation of signal density using the ECL detection method is limited, we routinely exposed chemiluminescent membranes to film for varying periods of time in order to obtain autoradiograms, which provided signals within the linear range for densitometry (Nishi et al., 1999c). Phospho-Thr[34] DARPP-32 bands were not quantified, because the ECL

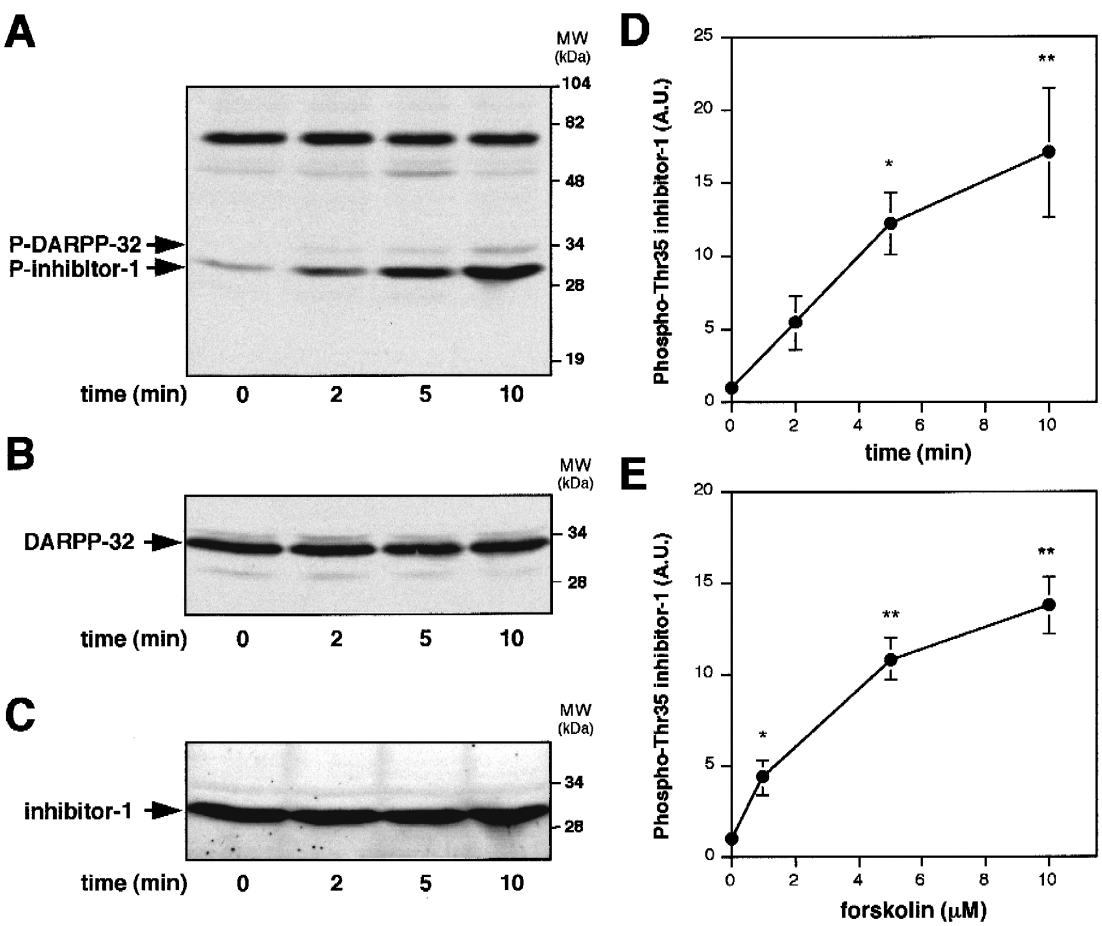

Fig. 2. Effect of forskolin on the levels of phosphorylated inhibitor-1 and DARPP-32. (A) Slices from renal medulla were incubated with forskolin (10 μM) for the indicated times. Phosphorylated inhibitor-1 and DARPP-32 at the cAMP-dependent protein kinase sites were both detected using a monoclonal antibody (mAb-23) against Thr[34]-phosphorylated DARPP-32. The phosphospecific antibody also detected a cross-reactive protein band at a molecular mass of ~75 kDa. Total DARPP-32 (B) and total inhibitor-1 (C) were detected on the same membrane. The amounts of phospho-Thr[35] inhibitor-1 in slices incubated with forskolin (10 μM) for the indicated times (D) and in slices incubated for 5 min in the presence of the indicated concentrations of forskolin (E) were quantified by densitometry, and the data were normalized to the values obtained with untreated slices. Data represent means ± S.E. for four to five experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with untreated slices; analysis of variance and Newman–Keuls test.

signals in control conditions, which were detectable after long exposure, were not strong enough for densitometric analysis.

2.3. Statistical analysis

Data were expressed as means ± S.E. Data were analyzed by analysis of variance and Newman–Keuls post hoc test or Student's *t*-test. Significance was considered to be $P < 0.05$.

3. Results

3.1. Expression of inhibitor-1 and DARPP-32 in renal medulla

The expression of inhibitor-1 and DARPP-32 in renal cortex and medulla was examined in comparison with the neostriatum. The amounts of inhibitor-1 and DARPP-32 in neostriatum have been reported to be 20 and 100 pmol/mg protein, respectively (Hemmings and Greengard, 1986; Hemmings et al., 1992). The levels of inhibitor-1, determined using the inhibitor-1 antibody (G185), and DARPP-32, determined using the DARPP-32 antibody (C24-5a), in mouse renal medulla were estimated to be 800% and 20% of that in neostriatum, respectively, and calculated to be 160 and 20 pmol/mg protein, respectively (Fig. 1). The levels of inhibitor-1 and DARPP-32 in mouse renal cortex were estimated to be 130% and 2% of that in neostriatum, respectively, and calculated to be 26 and 2 pmol/mg protein, respectively. The expression of inhibitor-1 and DARPP-32 in renal medulla was much higher than that in renal cortex. Furthermore, the level of inhibitor-1 expression in renal medulla was the highest in all tissues (Hemmings et al., 1992), indicating a critical role of inhibitor-1 in the regulation of protein phosphatase-1 activity in this tissue. For these reasons, we focused on studying the phosphorylation of inhibitor-1 and DARPP-32 in renal medulla.

3.2. Phosphorylation of inhibitor-1 and DARPP-32

The phosphorylation of inhibitor-1 and DARPP-32 by cAMP-dependent protein kinase was examined in slices of mouse renal medulla using a phosphorylation state-specific antibody that selectively detected the phosphorylation of both inhibitor-1 (Thr^{35}) and DARPP-32 (Thr^{34}) at the cAMP-dependent protein kinase-sites. Treatment of slices with forskolin (10 μM) increased the level of phosphorylated inhibitor-1 and DARPP-32 within 2 min of incubation, and was maximal at 10 min of incubation (Fig. 2A and D). The total amounts of DARPP-32 and inhibitor-1 protein were unaffected in each treatment conditions (Fig. 2B and C). The effect of forskolin on inhibitor-1 phosphorylation was maximal at a concentration of 10 μM with a half maximal effect at ~4 μM (Fig. 2E). It is also known that the cAMP-dependent protein kinase-sites of inhibitor-1 and DARPP-32 are substrates for cGMP-dependent protein kinase in vitro and in neuronal and nonneuronal cells (Towbin et al., 1979; Hemmings et al., 1984b; Snyder et al., 1992). To test the ability of cGMP-dependent protein kinase to phosphorylate inhibitor-1 and DARPP-32 in renal medulla, slices were treated with a cGMP analogue (Fig. 3). Treatment with the cAMP analogue, 8-bromo-cAMP (1 mM), increased the levels of phosphorylated inhibitor-1 by 23.0 ± 1.6-fold and of phosphorylated DARPP-32. In contrast, treatment with 8-bromo-cGMP (1 mM), which stimulated the phosphorylation of DARPP-32 in mouse neostriatal slices (data not shown), did not affect the phosphorylation of either inhibitor-1 or DARPP-32, indicating that cGMP-dependent protein kinase does not play a significant role in the phosphorylation of inhibitor-1 or DARPP-32 in slices of renal medulla.

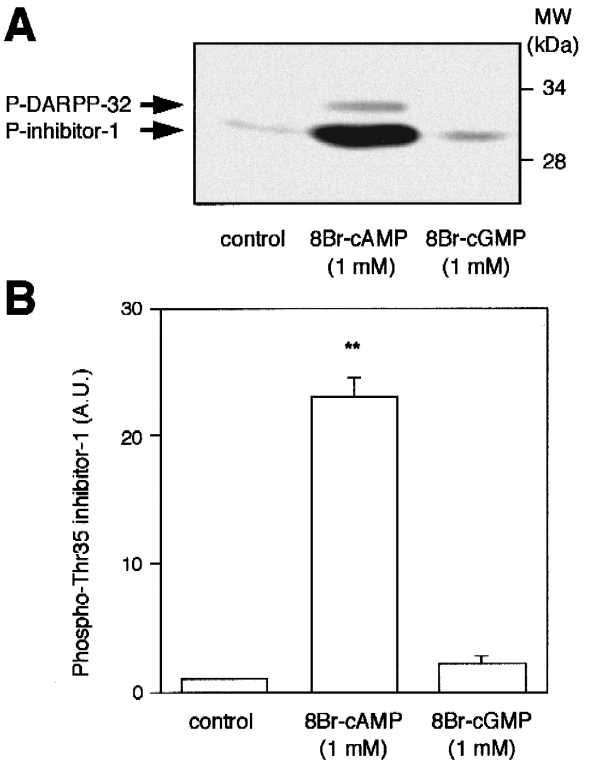

Fig. 3. Effect of cAMP and cGMP analogues on the levels of phosphorylated inhibitor-1 and DARPP-32. (A) Slices from renal medulla were incubated with a cAMP analogue, 8-bromo-cAMP (8Br-cAMP; 1 mM), or a cGMP analogue, 8-bromo-cGMP (8Br-cGMP; 1 mM), for 5 min. (B) The amount of phospho-Thr^{35} inhibitor-1 was quantified by densitometry, and the data were normalized to the values obtained with untreated slices. Data represent means ± S.E. for four to five experiments. $^{**}P < 0.01$ compared with control; analysis of variance and Newman–Keuls test.

3.3. Dephosphorylation of inhibitor-1 and DARPP-32

Phosphoforms of inhibitor-1 and DARPP-32 at cAMP-dependent protein kinase-sites are dephosphorylated by

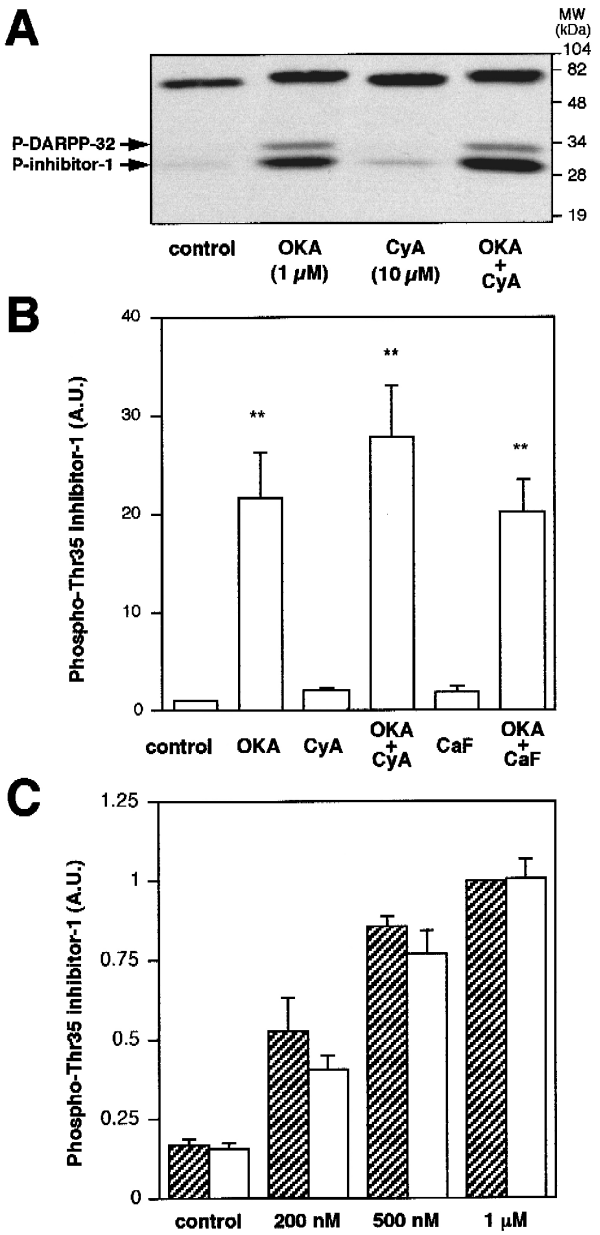

Fig. 4. Effect of protein phosphatase inhibitors on the levels of phosphorylated inhibitor-1 and DARPP-32. (A) Slices from renal medulla were incubated with okadaic acid (OKA; 1 μM) and/or cyclosporin A (CyA; 10 μM) for 60 min. Phosphorylated inhibitor-1 and DARPP-32 at the cAMP-dependent protein kinase sites were detected using a monoclonal antibody (mAb-23) against Thr^{34}-phosphorylated DARPP-32. The mobility of the ~75-kDa cross-reactive band was affected by okadaic acid. (B) Slices from renal medulla were incubated with okadaic acid (1 μM) and/or cyclosporin A (10 μM) for 60 min, or with okadaic acid for 60 min followed by incubation with Ca^{2+}-free/EGTA medium (CaF) for 20 min. The amount of phospho-Thr^{35} inhibitor-1 was quantified by densitometry, and the data were normalized to the values obtained with untreated slices. Data represent means ± S.E. for four experiments. $^{**}P < 0.01$ compared with control; analysis of variance and Newman–Keuls test. (C) Slices from renal medulla were incubated for 60 min with the indicated concentrations of okadaic acid (hatched bars) or calyculin A (open bars). The amount of phospho-Thr^{35} inhibitor-1 was quantified by densitometry, and the data were normalized to the values obtained with slices treated with okadaic acid (1 μM). Data represent means ± S.E. for four experiments.

protein phosphatase-2A and calcineurin, but not by protein phosphatase-1 or protein phosphatase-2C in vitro (Hemmings et al., 1984a; King et al., 1984). We recently reported that protein phosphatase-2A and calcineurin act synergistically to maintain a low level of phosphorylated DARPP-32 in neostriatal neurons (Nishi et al., 1999c). Dephosphorylation of inhibitor-1 and DARPP-32 was examined in slices of mouse renal medulla using protein phosphatase inhibitors. Treatment of slices with okadaic acid, a protein phosphatase-1/protein phosphatase-2A inhibitor, increased the level of phosphorylated inhibitor-1 and DARPP-32 (Fig. 4A and B). Okadaic acid acted in a dose-dependent manner with a half maximal effect on inhibitor-1 at ~200 nM (Fig. 4C). A maximal effect of okadaic acid was observed at a concentration of 1 μM, at which the level of inhibitor-1 was increased by 21.6 ± 4.5-fold. Calyculin A, another protein phosphatase-1/protein phosphatase-2A inhibitor that has a relatively larger effect than does okadaic acid on protein phosphatase-1 activity, had a dose-dependent effects similar to that of okadaic acid on the level of phosphorylated inhibitor-1 (Fig. 4C).

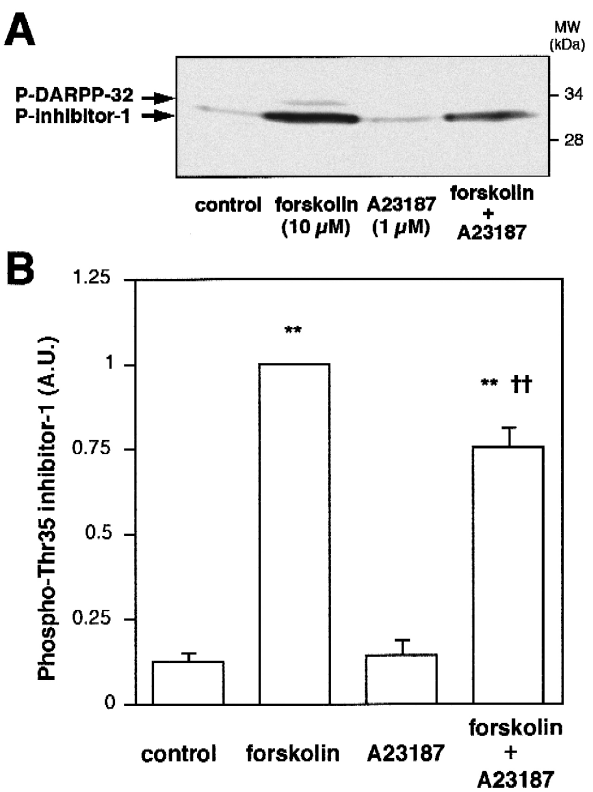

Fig. 5. Effect of Ca^{2+}-ionophore on the levels of phosphorylated inhibitor-1 and DARPP-32. (A) Slices from renal medulla were pre-incubated in the absence or presence of Ca^{2+}-ionophore, A23187 (1 μM), for 10 min, and then incubated with forskolin (10 μM) for an additional 5 min. (B) The amount of phospho-Thr^{35} inhibitor-1 was quantified by densitometry, and the data were normalized to the values obtained with forskolin-treated slices. Data represent means ± S.E. for three to four experiments. $^{**}P < 0.01$ compared with control; $^{††}P < 0.01$ compared with forskolin; analysis of variance and Newman–Keuls test.

Treatment of slices with cyclosporin A (10 μM), an inhibitor of calcineurin, did not affect the level of phosphorylated inhibitor-1 or DARPP-32 in the absence or presence of okadaic acid (1 μM) (Fig. 4A and B). Neither FK506 (1 μM), another potent inhibitor of calcineurin (data not shown), nor incubation with Ca^{2+}-free/EGTA medium (Fig. 4B), known to inactivate calcineurin in neurons (Nishi et al., 1999c), affected inhibitor-1 or DARPP-32 phosphorylation. To further examine the possible involvement of calcineurin in dephosphorylation of inhibitor-1 and DARPP-32, slices were treated with the Ca^{2+}-ionophore, A23187, to increase intracellular Ca^{2+} and activate calcineurin (Fig. 5). Treatment with A23187 (1 μM) did not affect the basal level of phosphorylated inhibitor-1 or DARPP-32, but reduced the forskolin (10 μM)-stimulated levels of phosphorylated inhibitor-1 by 24.7 ± 5.7% and of phosphorylated DARPP-32.

3.4. Effect of catecholamines on inhibitor-1 and DARPP-32 phosphorylation

It has been shown that Na^+ reabsorption in medullary thick ascending limb of Henle is physiologically regulated by catecholamines. The effect of activation of each catecholamine receptor on inhibitor-1 and DARPP-32 phosphorylation was examined in slices of mouse renal medulla. Treatment of slices with a β-adrenoceptor agonist, isoproterenol, increased the levels of phosphorylated inhibitor-1 and DARPP-32 (Fig. 6C). The effect of isoproterenol was observed within 1 min of incubation and reached a maximal level after 5 min of incubation (Fig. 6A). The effect of isoproterenol on inhibitor-1 phosphorylation was maximal at a concentration of 10 μM with a half-maximal effect at ~100 nM (Fig. 6B). To determine the subtype of β-adrenoceptors involved in the regulation of inhbitor-1

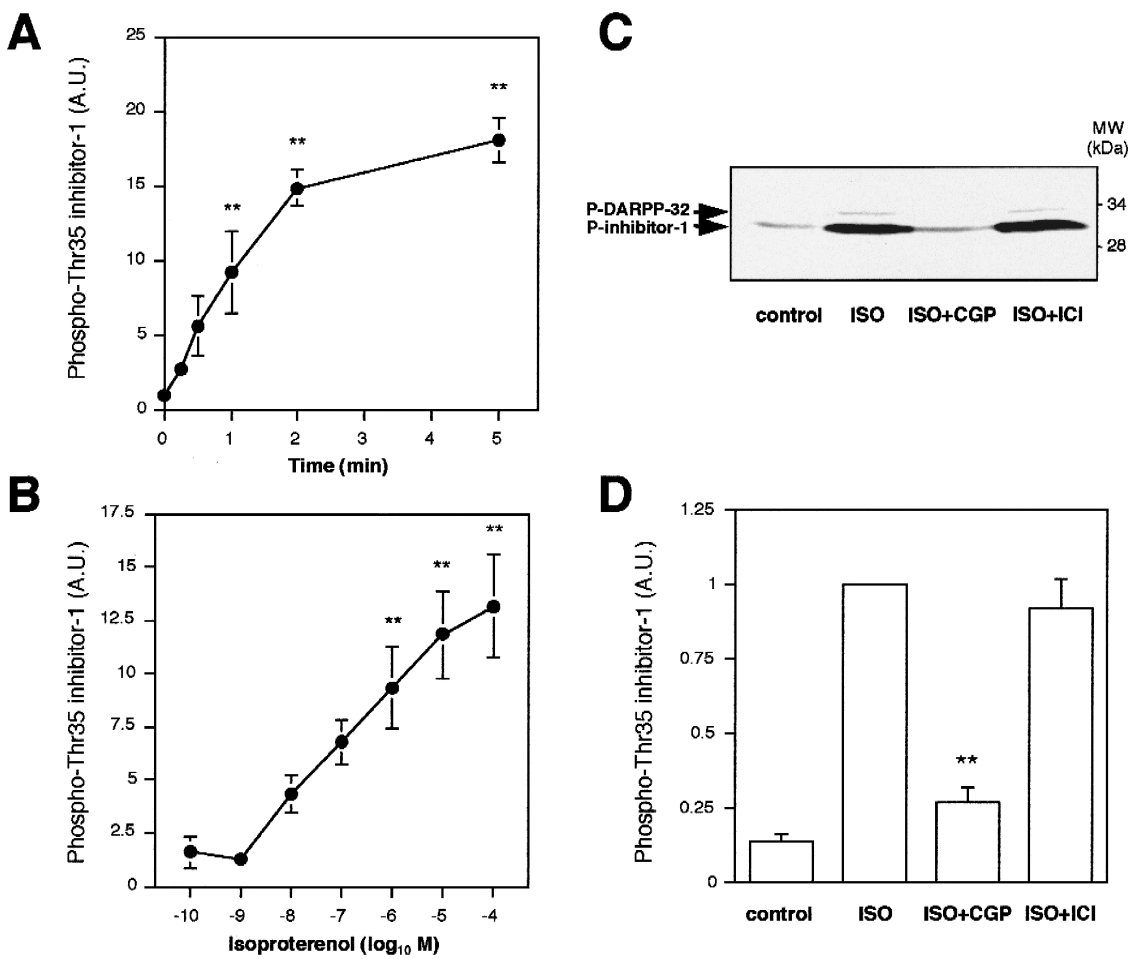

Fig. 6. Effect of β-adrenoceptor agonist on the level of phosphorylated inhibitor-1 and DARPP-32. Slices from renal medulla were incubated with a β-adrenoceptor agonist, isoproterenol (10 μM), for the indicated times (A) and with the indicated concentrations of isoproterenol for 5 min (B). The amount of phospho-Thr^{35} inhibitor-1 was quantified by densitometry, and the data were normalized to the values obtained with untreated slices. Data represent means ± S.E. for three to eight experiments. $^{**}P < 0.01$ compared with control; analysis of variance and Newman–Keuls test. (C and D) Slices from renal medulla were incubated with isoproterenol (ISO; 1 μM) for 5 min in the presence of a β_1-adrenoceptor antagonist, CGP20712A (CGP; 1 μM), or a β_2-adrenoceptor antagonist, ICI118551 (ISI; 1 μM). The amount of phospho-Thr^{35} inhibitor-1 was quantified by densitometry, and the data were normalized to the values obtained with isoproterenol-treated slices. Data represent means ± S.E. for four experiments. $^{**}P < 0.01$ compared with isoproterenol alone; analysis of variance and Newman–Keuls test.

and DARPP-32 phosphorylation, slices were treated with a β_1-adrenoceptor selective antagonist, CGP20712A, or a β_2-adrenoceptor selective antagonist, ICI118551 (Fig. 6C and D). The effect of isoproterenol (10 μM) was completely abolished by CGP20712A (1 μM), but not by ICI118551 (1 μM), indicating that isoproterenol activates β_1-adrenoceptors, increases cAMP levels and cAMP-dependent protein kinase activity, and stimulates the phosphorylation of inhibitor-1 and DARPP-32 in renal medulla. In contrast, treatment of slices with an α-adrenoceptor agonist, oxymetazoline (10 μM), did not affect the basal or isoproterenol-stimulated level of phosphorylated inhibitor-1 (Fig. 7A). Treatment with α_1-adrenoceptor selective agonist, cirazoline, an α_2-adrenoceptor selective agonist, UK14304, or cirazoline plus UK14304 had no effect on inhibitor-1 phosphorylation (data not shown).

The effect of activation of dopamine D1 receptors on inhibitor-1 and DARPP-32 phosphorylation was also examined (Fig. 7B and C). Treatment of slices with a dopamine D1 receptor agonist, SKF81297 (1 μM), slightly increased the levels of phosphorylated inhibitor-1 by 2.18 ± 0.21-fold and of phosphorylated DARPP-32. Stimulation of inhibitor-1 phosphorylation by SKF81297 was much weaker than that by isoproterenol.

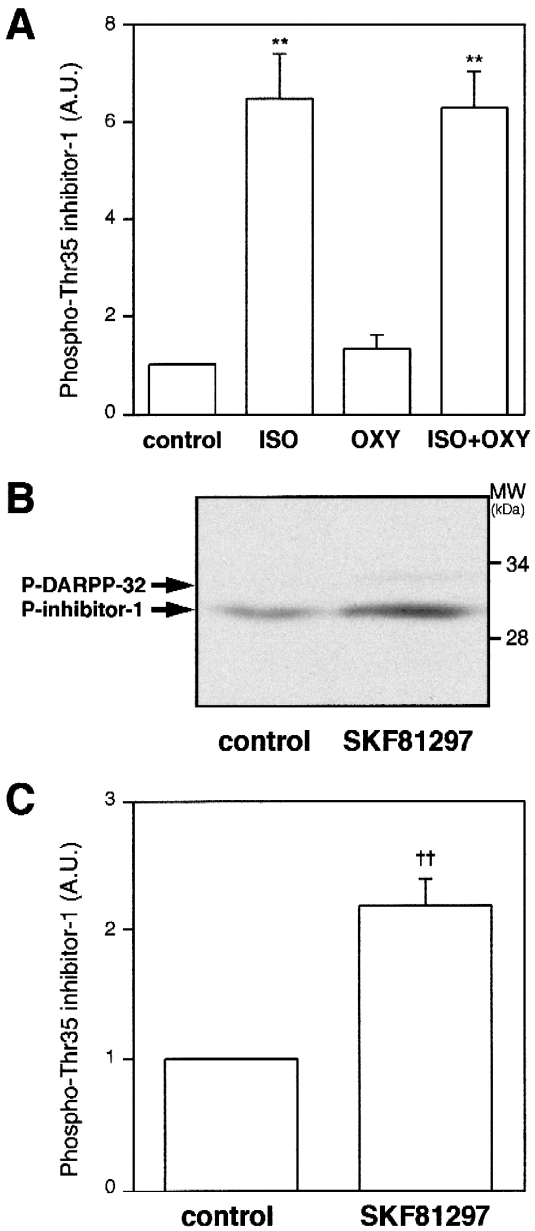

Fig. 7. Effects of α-adrenoceptor and dopamine D1 receptor agonists on the level of phosphorylated inhibitor-1 and DARPP-32. (A) Slices from renal medulla were incubated for 5 min with an α-adrenoceptor agonist, oxymetazoline (OXY; 10 μM), in the absence or presence of a β-adrenoceptor agonist, isoproterenol (ISO; 10 μM). The amount of phospho-Thr^{35} inhibitor-1 was quantified by densitometry, and the data were normalized to the values obtained with untreated slices. Data represent means ± S.E. for six to ten experiments. $^{**}P < 0.01$ compared with untreated slices; analysis of variance and Newman–Keuls test. (B and C) Slices from renal medulla were incubated for 5 min with a dopamine D1 receptor agonist, SKF81297 (1 μM). The amount of phospho-Thr^{35} inhibitor-1 was quantified by densitometry, and the data were normalized to the values obtained with untreated slices. Data represent means ± S.E. for four experiments. $^{††}P < 0.01$ compared with control; Student's *t*-test.

4. Discussion

The results of this study demonstrate that, in renal medulla, the state of phosphorylation of inhibitor-1 and DARPP-32 is dynamically regulated by the balance of phosphorylation by cAMP-dependent protein kinase and dephosphorylation by protein phosphatase-2A (Fig. 8). Activation of cAMP-dependent protein kinase by forskolin or cAMP analogue or by stimulation of β_1-adrenoceptors or dopamine D1 receptors leads to phosphorylation of inhibitor-1 and DARPP-32, resulting in inhibition of protein phosphatase-1 and an increase in the state of phosphorylation of protein phosphatase-1 substrate. Inhibitor-1 and DARPP-32 at cAMP-dependent protein kinase sites are also substrates for phosphorylation by cGMP-dependent protein kinase in vitro and in intact tissues (Hemmings et al., 1984b; Snyder et al., 1992; Tsou et al., 1993). However, in renal medulla, a cGMP analogue did not affect the phosphorylation of inhibitor-1 or DARPP-32. These data are in agreement with a report showing very low expression of cGMP-dependent protein kinase in renal medulla (Gambaryan et al., 1996). These results indicate that inhibitor-1 and DARPP-32 function as a signaling cascade for cAMP-dependent protein kinase, but not for cGMP-dependent protein kinase, in renal medulla.

Phosphorylated forms of inhibitor-1 and DARPP-32 at the cAMP-dependent protein kinase sites are substrates for protein phosphatase-2A and calcineurin but not for protein phosphatase-1 or protein phosphatase-2C in vitro (Hemmings et al., 1984a; King et al., 1984). In slices from renal medulla, the effect of okadaic acid on inhibitor-1 phosphorylation was maximal at 1 μM with an IC_{50} of ~200 nM. We previously reported that okadaic acid at a concentration of 1 μM completely inhibits protein phosphatase-2A

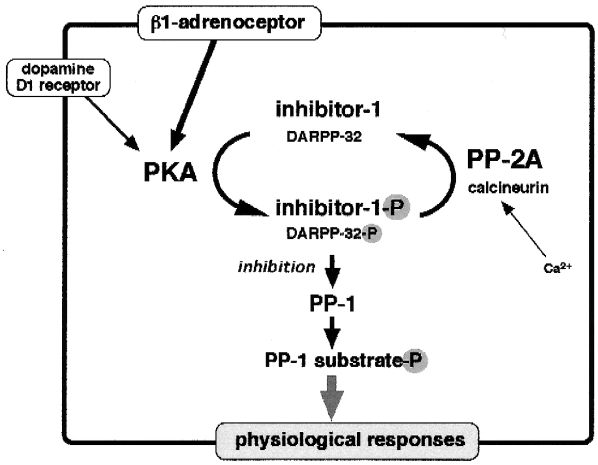

Fig. 8. Model illustrating the regulation of phosphorylation/dephosphorylation of inhibitor-1 and DARPP-32 in renal medulla. Activation of cAMP-dependent protein kinase (PKA) by β_1-adrenoceptors and dopamine D1 receptors, but not of cGMP-dependent protein kinase, stimulates the phosphorylation of inhibitor-1 at Thr^{35} (inhibitor-1-P) and DARPP-32 at Thr^{34} (DARPP-32-P). Phospho-Thr^{35} inhibitor-1 and phospho-Thr^{34} DARPP-32 are dephosphorylated mainly by protein phosphatase-2A and possibly by calcineurin when it is activated. The state of phosphorylation of inhibitor-1 and DARPP-32 is dynamically regulated by the balance of phosphorylation by cAMP-dependent protein kinase and dephosphorylation by protein phosphatase-2A. Phosphorylation of inhibitor-1 and DARPP-32 at cAMP-dependent protein kinase-sites converts them to potent inhibitors of protein phosphatase-1, resulting in the increase in the phosphorylation levels and the altered functions of its substrate proteins.

activity but it inhibits protein phosphatase-1 activity by only 30% in slices from neostriatum, where 100–1000 times higher concentrations of okadaic acid are required than in in vitro conditions (Ishihara et al., 1989; Nishi et al., 1999c). These results, therefore, indicate that the effect of okadaic acid is mediated through inhibition of protein phosphatase-2A activity. In addition, calyculin A, which has a relatively greater effect on protein phosphatase-1 activity than does okadaic acid, had dose-dependent effects similar to okadaic acid on inhibitor-1 and DARPP-32 phosphorylation, supporting a significant role of protein phosphatase-2A in the dephosphorylation of inhibitor-1 and DARPP-32.

The dephosphorylation of inhibitor-1 and DARPP-32 by calcineurin was negligible under basal conditions, since neither a high concentration of cyclosporin A (10 μM) nor Ca^{2+}-free/EGTA medium affected the dephosphorylation of inhibitor-1 and DARPP-32. These results are in contrast to the role of calcineurin in neostriatum, where the expression of calcineurin is high compared to renal tissues (Goto et al., 1986; Tumlin et al., 1995), and where calcineurin makes a greater contribution to the dephosphorylation of DARPP-32 than does protein phosphatase-2A (Nishi et al., 1999c). However, when calcineurin was activated by increasing the concentration of intracellular Ca^{2+} with Ca^{2+}-ionophore, calcineurin slightly reduced the forskolin-stimulated phosphorylation of inhibitor-1 and DARPP-32. Inhibitor-1 and DARPP-32 are dephosphorylated mainly by protein phosphatase-2A under basal conditions and slightly by calcineurin in response to stimuli that increase the concentration of intracellular Ca^{2+}. Thus, the dephosphorylation of inhibitor-1 and DARPP-32 is regulated by protein phosphatases in a tissue-specific manner.

The expression of inhibitor-1 in renal medulla was estimated to be 160 pmol/mg protein, 8-fold higher than that of DARPP-32 (20 pmol/mg protein), suggesting a greater relative importance of inhibitor-1 as an inhibitor of protein phosphatase-1 in this tissue. In addition to phosphorylation by cAMP-dependent protein kinase, inhibitor-1 (at Ser^{67}) and DARPP-32 (at Thr^{75}) are each phosphorylated by Cyclin-dependent kinase (Bibb et al., 1999; Huang and Paudel, 2000). Phosphorylation of DARPP-32, but not inhibitor-1, by Cyclin-dependent kinase converts it into a competitive inhibitor of cAMP-dependent protein kinase, indicating that DARPP-32 and inhibitor-1 have different functional roles. In neostriatal neurons, DARPP-32, phosphorylated by Cdk5, is involved in the modulation of the efficacy of dopamine signaling (Bibb et al., 1999; Nishi et al., 2000). We found that DARPP-32 at Thr^{75} is highly phosphorylated in renal medulla (Nishi, unpublished observations). These findings suggest that, in spite of low expression, DARPP-32 plays a specific role in the regulation of tubular functions.

Slice preparations of renal medulla were used in this study. It has been reported that inhibitor-1 and DARPP-32 are selectively expressed in the nephron segment of thick ascending limb using techniques of immunohistochemistry and in situ hybridization (Meister et al., 1989). Because of the selective expression of inhibitor-1 and DARPP-32 in medullary thick ascending limb of Henle, changes in the state of phosphorylation of those proteins observed in slices can be attributed to the changes in medullary thick ascending limb of Henle. Medullary thick ascending limb of Henle plays an important role in determining the rate of Na^+ reabsorption. Changes in the state of phosphorylation of inhibitor-1 and DARPP-32 modulate the activity of protein phosphatase-1, resulting in changes in the phosphorylation level of its substrates. The inhibition of protein phosphatase-1 activity by phospho-Thr^{34} DARPP-32 peptide (Aperia et al., 1991) or protein phosphatase-1 inhibitors (calyculin A and okadaic acid) (Li et al., 1995; Slobodyansky et al., 1995) has been reported to inhibit the activity of Na^+,K^+-ATPase by increasing the phosphorylation of Na^+,K^+-ATPase at the cAMP-dependent protein kinase site (Fisone et al., 1994; Cheng et al., 1997) or by the phosphorylation of intermediate protein(s) that in turn modulate the activity of Na^+,K^+-ATPase as previously proposed (Feschenko and Sweadner, 1995; Nishi et al., 1999a). Direct phosphorylation of Na^+,K^+-ATPase at the cAMP-dependent protein kinase site may not be involved in the inhibition of enzyme activity, since the phosphorylation of Na^+,K^+-ATPase at the cAMP-dependent protein kinase site, determined using a phospho-specific antibody

against phospho-Ser^{943} (Fisone et al., 1994), was not modulated by the treatment of slices with forskolin (Higuchi and Nishi, unpublished observations)

Na^+ reabsorption from renal tubule is regulated by various hormones and neurotransmitters. Sympathetic nerve terminals densely innervate renal tubules and regulate the rate of Na^+ reabsorption (DiBona and Kopp, 1997). Four subtypes of β-adrenoceptors (referred to as β_1, β_2, β_3 and β_4) have been identified (Kaumann, 1997), and β_1- and β_2-adrenoceptors are expressed in renal tubules (Elalouf et al., 1993; DiBona and Kopp 1997). Activation of β_1-adrenoceptors, but not β_2-adrenoceptors, stimulated the phosphorylation of inhibitor-1 and DARPP-32. In agreement with our results, a study in dissected segments of medullary thick ascending limb of Henle revealed that the expression of β_1-adrenoceptors is high but that of β_2-adrenoceptors is low (Elalouf et al., 1993). Activation of α-adrenoceptors did not affect the phosphorylation of inhibitor-1 and DARPP-32 in basal and β-adrenoceptor agonist-stimulated conditions. Aperia et al. (1992) reported that activation of α-adrenoceptors increases the intracellular Ca^{2+} concentration and activates calcineurin in proximal convoluted tubule, resulting in the activation of Na^+,K^+-ATPase. The lack of effect of an α-adrenoceptor agonist on inhibitor-1and DARPP-32 phosphorylation in medullary thick ascending limb of Henle could be explained by a low expression of α-adrenoceptors (DiBona and Kopp, 1997) or by a small contribution of calcineurin in dephosphorylation of inhibitor-1.

Dopamine functions as a natriuretic hormone by inhibiting Na^+,K^+-ATPase activity in tubular segments including medullary thick ascending limb of Henle (Aperia, 2000). In contrast, activation of β-adrenoceptors has been reported to stimulate Na^+ reabsorption in medullary thick ascending limb of Henle (Bailly et al., 1990; Morgunov et al., 1993). We found in this study that activation of dopamine D1 receptors, as well as activation of β-adrenoceptors, stimulated the phosphorylation of inhibitor-1 and DARPP-32, indicating that effects of β-adrenoceptors and dopamine D1 receptors, which have opposing effects on Na^+ reabsorption, are mediated through the same signaling cascade: activation of cAMP-dependent protein kinase, phosphorylation of inhibitor-1 and DARPP-32, and inhibition of protein phosphatase-1. Besides the regulation of protein phosphatase-1 activity by inhibitor-1 and DARPP-32, it is possible that additional signaling systems, which are activated by each receptor or by other receptor subtypes, are involved to induce the natriuretic or antinatriuretic effects. For example, dopamine D1 receptors have been reported to couple to phospholipase C and phospholipase A2 (Satoh et al., 1992; Yu et al., 1996 37; Hussain and Lokhandwala, 1998). Furthermore, Gao et al. (1992) reported that stimulatory or inhibitory effects of β-adrenoceptors on Na^+,K^+-ATPase activity are dependent on the concentration of intracellular Ca^{2+}. The importance of intracellular Ca^{2+} is supported by the observation that the effects of cAMP-dependent protein kinase and protein kinase C on Na^+,K^+-ATPase activity are dependent on the concentration of intracellular Ca^{2+} (Cheng et al., 1999). The complete mechanisms by which the activation of cAMP-dependent protein kinase and the inhibition of protein phosphatase-1 induce either inhibition or stimulation of Na^+ reabsorption in medullary thick ascending limb of Henle remains to be elucidated.

Acknowledgements

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (to A.N.). The authors thank Yukako Terasaki for excellent technical assistance.

References

Aperia, A.C., 2000. Intrarenal dopamine: a key signal in the interactive regulation of sodium metabolism. Annu. Rev. Physiol. 62, 621–647.

Aperia, A., Fryckstedt, J., Svensson, L.-B., Hemmings, H.C. Jr., Nairn, A.C., Greengard, P., 1991. Phosphorylated Mr 32,000 dopamine- and cAMP-regulated phosphoprotein inhibits Na^+,K^+-ATPase activity in renal tubule cells. Proc. Natl. Acad. Sci. U. S. A. 88, 2798–2801.

Aperia, A., Ibarra, F., Svensson, L.-B., Klee, C., Greengard, P., 1992. Calcineurin mediates α-adrenergic stimulation of Na^+,K^+-ATPase activity in renal tubule cells. Proc. Natl. Acad. Sci. U. S. A. 89, 7394–7397.

Bailly, M., Imbert-Teboul, M., Roinel, N., Animel, C., 1990. Isoproterenol increases Ca, Mg, and NaCl reabsorption in mouse thick ascending limb. Am. J. Physiol. 258, F1224–F1231.

Begiun, P., Beggah, A., Cotecchia, S., Geering, K., 1996. Adrenergic, dopaminergic, and muscarinic receptor stimulation leads to PKA phosphorylation of Na–K-ATPase. Am. J. Physiol. 270, C131–C137.

Bibb, J.A., Snyder, G.L., Nishi, A., Yan, Z., Meijer, L., Fienberg, A.A., Tsai, L.H., Kwon, Y.T., Girault, J.A., Czernik, A.J., Huganir, R.L., Hemmings, H.C. Jr., Nairn, A.C., Greengard, P., 1999. Phosphorylation of DARPP-32 by Cdk 5 modulates dopamine signaling in neurons. Nature 402, 669–671.

Cheng, X.-J., Fisone, G., Aizman, O., Aizman, R., Levenson, R., Greengard, P., Aperia, A., 1997. cAMP-dependent protein kinase-mediated phosphorylation and inhibition of Na^+,K^+-ATPase in response to β-adrenergic hormone. Am. J. Physiol. 273, C893–C901.

Cheng, S.-X., Aizman, O., Nairn, A.C., Greengard, P., Aperia, A., 1999. $[Ca^{2+}]_i$ determines the effects of protein kinase A and C on activity of rat renal Na^+,K^+-ATPase. J. Physiol. 518, 37–46.

DiBona, G.F., Kopp, U.C., 1997. Neural control of renal function. Physiol. Rev. 77, 75–197.

Elalouf, J.-M., Buhler, J.-M., Tessiot, C., Bellanger, A.-C., Dublineau, I., de Rouffignac, C., 1993. Predominant expression of β1-adrenergic receptor in the thick ascending limb of rat kidney. J. Clin. Invest. 91, 264–272.

Feschenko, M.S., Sweadner, K.J., 1995. Structural basis for species-specific differences in the phosphorylation of Na,K-ATPase by protein kinase C. J. Biol. Chem. 270, 14072–14077.

Fienberg, A.A., Hiroi, N., Mermelstein, P., Song, W.-J., Snyder, G.L., Nishi, A., Cheramy, A., O'Callaghan, J.P., Miller, D., Cole, D., Corbett, R., Haile, C., Cooper, D., Onn, S., Grace, A.A., Ouimet, C., White, F.J., Hyman, S.E., Surmeier, D.J., Girault, J.A., Nestler, E., Greengard, P., 1998. DARPP-32, regulator of the efficacy of dopaminergic neurotransmission. Science 281, 838–842.

Fisone, G., Cheng, S.X.-J., Nairn, A.C., Czernik, A.J., Hoog, J.-O., Bertorello, A.M., Kaiser, R., Bergman, T., Jornvall, H., Aperia, A., Greengard, P., 1994. Identification of the phosphorylation site for cAMP-dependent protein kinase on Na^+,K^+-ATPase and effects of site-directed mutagenesis. J. Biol. Chem. 269, 9368–9373.

Gambaryan, S., Hausler, C., Markert, T., Pohler, D., Jarchau, T., Walter, U., Haase, W., Kurtz, A., Lohmann, S.M., 1996. Expression of type II cGMP-dependent protein kinase in rat kidney is regulated by dehydration and correlated with renin gene expression. J. Clin. Invest. 98, 662–670.

Gao, B.J., Mathias, R.T., Cohen, I.S., Baldo, G.J., 1992. Isoprenaline, Ca^{2+} and the Na^+–K^+ pump in guinea-cAMP-dependent protein kinase ventricular myocytes. J. Physiol. 449, 689–704.

Goto, S., Matsukado, Y., Mihara, Y., Inoue, N., Miyamoto, E., 1986. The distribution of calcineurin in rat brain by light and electron microscopic immunocytochemistry and enzyme assay. Brain Res. 397, 161–172.

Greengard, P., Allen, P.B., Nairn, A.C., 1999. Beyond the dopamine receptor: the DARPP-32/protein phosphatase-1 cascade. Neuron 23, 435–447.

Hemmings, H.C. Jr., Greengard, P., 1986. DARPP-32, a dopamine- and adenosine 3′:5′-monophosphate-regulated phosphoprotein: regional, tissue, and phylogenetic distribution. J. Neurosci. 6, 1469–1481.

Hemmings, H.C. Jr., Greengard, P., Tung, H.Y.L., Cohen, P., 1984a. DARPP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase-1. Nature 310, 503–505.

Hemmings, H.C. Jr., Nairn, A.C., Greengard, P., 1984b. DARPP-32, a dopamine- and adenosine 3′:5′-monophosphate-regulated phosphoprotein enriched in dopamine-innervated brain regions: II. Comparison of the kinetics of phosphorylation of DARPP-32 and phosphatase inhibitor-1. J. Biol. Chem. 259, 14491–14497.

Hemmings, H.C. Jr., Girault, J.-A., Nairn, A.C., Bertuzzi, G., Greengard, P., 1992. Distribution of protein phosphatase inhibitor-1 in brain and peripheral tissues of various species: comparison with DARPP-32. J. Neurochem. 59, 1053–1061.

Huang, K.X., Paudel, H.K., 2000. Ser^{67}-phosphorylated inhibitor 1 is a potent protein phosphatase 1 inhibitor. Proc. Natl. Acad. Sci. U. S. A. 97, 5824–5829.

Hussain, T., Lokhandwala, M.F., 1998. Renal dopamine receptor function in hypertension. Hypertension 32, 187–197.

Ishihara, H., Martin, B.L., Brautigan, D.L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K., Uemura, D., Hartshorne, D.J., 1989. Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. Biochem. Biophys. Res. Commun. 159, 871–877.

Kaumann, A.J., 1997. Four β-adrenoceptor subtypes in the mammalian heart. Trends Pharmacol. Sci. 18, 70–76.

King, M.M., Huang, C.Y., Chock, P.B., Nairn, A.C., Hemmings, H.C. Jr., Chan, K.-F.J., Greengard, P., 1984. Mammalian brain phosphoproteins as substrates for calcineurin. J. Biol. Chem. 259, 8080–8083.

Li, D., Aperia, A., Celsi, G., da Cruz e Silva, E.F., Greengard, P., Meister, B., 1995. Protein phosphatase-1 in the kidney: evidence for a role in the regulation of medullary Na^+,K^+-ATPase. Am. J. Physiol. 269, F673–F680.

Meister, B., Fryckstedt, J., Schalling, M., Cortes, R., Hokfelt, T., Aperia, A., Hemmings, H.C. Jr., Nairn, A.C., Ehrlich, M., Greengard, P., 1989. Dopamine- and cAMP-regulated phosphoprotein (DARPP-32) and dopamine DA1 agonist-sensitive Na^+,K^+-ATPase in renal tubule cells. Proc. Natl. Acad. Sci. U. S. A. 86, 8068–8072.

Morgunov, N.S., You, Y.-D., Hirsch, D.J., 1993. Response of mouse proximal straight tubule and medullary thick ascending limb to β-agonist. J. Am. Soc. Nephrol. 4, 1151–1158.

Nishi, A., Fisone, G., Snyder, G.L., Dulubova, I., Aperia, A., Nairn, A.C., Greengard, P., 1999a. Regulation of Na^+,K^+-ATPase isoforms in rat neostriatum by dopamine and protein kinase C. J. Neurochem. 73, 1492–1501.

Nishi, A., Snyder, G.L., Fienberg, A.A., Fisone, G., Aperia, A., Nairn, A.C., Greengard, P., 1999b. Requirement for DARPP-32 in mediating effect of dopamine D2 receptor activation. Eur. J. Neurosci. 11, 2589–2592.

Nishi, A., Snyder, G.L., Nairn, A.C., Greengard, P., 1999c. Role of calcineurin and protein phosphatase-2A in the regulation of DARPP-32 dephosphorylation in neostriatal neurons. J. Neurochem. 72, 2015–2021.

Nishi, A., Bibb, J.A., Snyder, G.L., Higashi, H., Nairn, A.C., Greengard, P., 2000. Amplification of dopaminergic signaling by a positive feedback loop. Proc. Natl. Acad. Sci. U. S. A., in press.

Satoh, T., Cohen, H.T., Katz, A.I., 1992. Intracellular signaling in the regulation of renal Na–K-ATPase: I. Role of cyclic AMP and phospholipase A2. J. Clin. Invest. 89, 1496–1500.

Slobodyansky, E., Aoki, Y., Gaznavi, A.K.M., Aviles, D.H., Fildes, R.D., Jose, P.A., 1995. Dopamine and protein phosphatase activity in renal proximal tubules. Am. J. Physiol. 268, F279–F284.

Snyder, G.L., Girault, J.-A., Chen, J.Y.C., Czernik, A.J., Kebabian, J.W., Nathanson, J.A., Greengard, P., 1992. Phosphorylation of DARPP-32 and protein phosphatase inhibitor-1 in rat choroid plexus: regulation by factors other than dopamine. J. Neurosci. 12, 3071–3083.

Towbin, H., Staehlin, T., Gordon, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354.

Tsou, K., Snyder, G.L., Greengard, P., 1993. Nitric oxide/cGMP pathway stimulates phosphorylation of DARPP-32, a dopamine- and cAMP-regulated phosphoprotein, in the substantia nigra. Proc. Natl. Acad. Sci. U. S. A. 90, 3462–3465.

Tumlin, J.A., Someren, J.T., Swanson, C.E., Lea, J.P., 1995. Expression of calcineurin activity and α-subunit isoforms in specific segments of the rat nephron. Am. J. Physiol. 269, F558–F563.

Yu, P.-Y., Eisner, G.M., Yamaguchi, I., Mouradian, M.M., Felder, R.A., Jose, P.A., 1996. Dopamine D1A receptor regulation of phospholipase C isoform. J. Biol. Chem. 271, 19503–19508.