Stimulation of ouabain-sensitive $^{86}Rb^+$ uptake and Na^+,K^+-ATPase α-subunit phosphorylation by a cAMP-dependent signalling pathway in intact cells from rat kidney cortex

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Abstract **We investigated in intact cortical kidney tubules the role of PKA-mediated phosphorylation in the short-term control of Na^+,K^+-ATPase activity. The phosphorylation level of Na^+,K^+-ATPase was evaluated after immunoprecipitation of the enzyme from ^{32}P-labelled cortical tubules and the cation transport activity of Na^+,K^+-ATPase was measured by ouabain-sensitive $^{86}Rb^+$ uptake. Incubation of cells with cAMP analogues (8-bromo-cAMP, dibutyryl-cAMP) or with forskolin plus 3-isobutyl-1-methylxanthine increased the phosphorylation level of the Na^+,K^+-ATPase α-subunit and stimulated ouabain-sensitive $^{86}Rb^+$ uptake. Inhibition of PKA by H-89 blocked the effects of dibutyryl-cAMP on both phosphorylation and $^{86}Rb^+$ uptake processes. The results suggest that phosphorylation by PKA stimulates the Na^+,K^+-ATPase activity.**

Key words: Na^+,K^+-ATPase; Rubidium uptake; Protein phosphorylation; cAMP analog; Forskolin; Rat kidney cortex

1. Introduction

Na^+,K^+-ATPase (EC 3.6.1.37) is a key enzyme which maintains the transmembrane gradients of Na^+ and K^+ in every mammalian cell. In the kidney, these ion gradients allow the reabsorption of water and solutes along the nephron. Therefore, Na^+,K^+-ATPase activity should be dynamically regulated to preserve body fluid homeostasis. Besides Na^+,K^+-ATPase activity regulation by several factors such as intracellular sodium, extracellular potassium, ATP, and cardiac glycosides, its control by various hormones, neurotransmitters and growth factors has also been demonstrated. Most of these molecules work through signal transduction cascades involving protein kinase activation, raising the question of Na^+,K^+-ATPase regulation by phosphorylation processes. Evidence for short-term regulation of Na^+,K^+-ATPase by phosphorylation is emerging. To date, one of the major recognized intracellular signalling pathways involves activation of protein kinase C (PKC). We recently demonstrated that in intact cells of rat cortical kidney, activation of PKC induces stimulation of the Na^+,K^+-ATPase transport activity associated with an increase in the phosphorylation level of its α-subunit [1]. Another major signalling pathway involves the modulation of adenylyl cyclase activity and the alteration of intracellular cAMP levels. An increase in cAMP level results in activation of the cAMP-dependent protein kinase (PKA). There is a bulk of evidence for short-term regulation of Na^+,K^+-ATPase by conditions that activate PKA. However, controversial PKA activation effects on the ion transport capacity of the Na^+,K^+-ATPase have been reported in experiments performed in different tissues as well as in the same tissue. For instance, exposure of renal proximal tubules to cAMP analogues reduces [2], increases [3] or does not affect [4,5] Na^+,K^+-ATPase activity. Experimental conditions could be one of the reasons for these discrepancies by analogy to what was recently demonstrated for the effects of PKC activation on Na^+,K^+-ATPase activity in rat PCT [6], where the opposite effect was obtained according to the cellular oxygenation status.

PKA-mediated phosphorylation of the Na^+,K^+-ATPase α-subunit is a possible pathway for the effect of cAMP in proximal cells. Recent studies suggest that the α-subunit of Na^+,K^+-ATPase is a target for regulatory phosphorylation by protein kinase A: PKA phosphorylates Na^+,K^+-ATPase α-subunit in vitro [7] and mutation of the single PKA-phosphorylation site abolished the PKA-mediated phosphorylation of Na^+,K^+-ATPase in transfected COS cells [8,9]. However, PKA-mediated phosphorylation of the renal Na^+,K^+-ATPase has not been demonstrated in non-transfected intact cells. The physiological link between an increased phosphorylation level and the activity of the Na^+,K^+-ATPase is another controversial point. Several studies reported that the increase in phosphorylation is accompanied by stimulation of the enzyme activity [10,11], but others showed an inhibition of Na^+,K^+-ATPase activity after its phosphorylation [7]. These discrepancies can be explained, at least in part, by the experimental conditions, i.e. the results obtained with purified enzyme are influenced by the in vitro phosphorylation conditions, requiring the presence of Triton X-100, a detergent known to inhibit Na^+,K^+-ATPase activity. It has been demonstrated in isolated membranes from COS cells transfected with cDNAs encoding the rat α_1-subunit that forskolin plus IBMX decreases the V_{max} of the wild-type enzyme but not that of the enzyme mutated in the PKA phosphorylation site [8]. This result favors an inhibitory effect of the activation of PKA pathway on Na^+,K^+-ATPase activity, but does not rule out the possibility of a cytoplasm factor intervening in physiological conditions, lost during the permeabilisation step.

In this context, the aim of the present work was to investigate the role of PKA in the control of Na^+,K^+-ATPase activity in intact cortical kidney cells. For this purpose, we analyzed the relationship between the phosphorylation level of the Na^+,K^+-ATPase and its cation transport activity in re-

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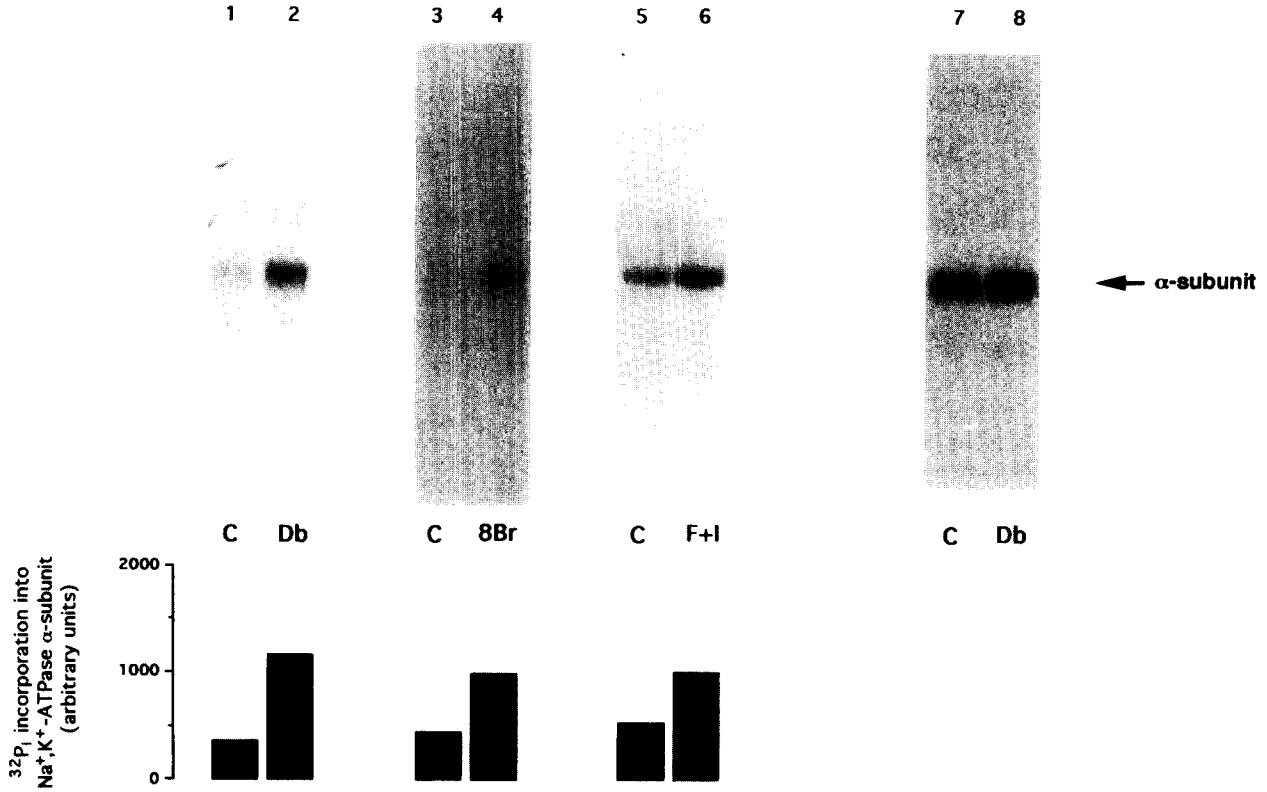

Fig. 1. Phosphorylation level of the Na^+,K^+-ATPase α-subunit under basal and PKA activation conditions. Suspensions of ^{32}P-labelled tubules were incubated at 37°C for 15 min in the absence (C, control) or presence of PKA activators: 1 mM Db-cAMP (Db), 1 mM 8Br-cAMP (8Br) or with 0.01 mM forskolin plus 0.1 mM IBMX (F+I). After Na^+,K^+-ATPase immunoprecipitation, immune complexes were analyzed by 5–12% SDS-PAGE and transferred to PVDF membranes. (Upper panel) Representative autoradiograms of immunoprecipitated Na^+,K^+-ATPase from control tubules (lanes 1,3,5) or PKA activator-treated tubules (lanes 2,4,6) from 4–11 independent experiments. Lanes 7–8, representative immunoblot of the α-subunit of Na^+,K^+-ATPase with an anti-rat α_1-subunit monoclonal antibody (McK1). (Lower panel) Quantification of $^{32}P_i$ incorporation into α-subunit shown in upper panel expressed as arbitrary units. Data are means of triplicate measurement of each band intensity.

sponse to modulation of PKA activity. We show that activation of the cAMP-dependent protein kinase enhances Na^+,K^+-ATPase activity through an increased phosphorylation level of the catalytic α-subunit of the pump.

2. Materials and methods

2.1. Preparation of cortical tubules suspensions

Kidneys from male Wistar rats (body weight 130–150 g) were perfused with ice-cold incubation solution (120 mM NaCl, 5 mM RbCl, 4 mM $NaHCO_3$, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.2 mM NaH_2PO_4, 0.15 mM Na_2HPO_4, 5 mM glucose, 10 mM lactate, 1 mM pyruvate, 4 mM essential and non-essential amino acids, 0.03 mM vitamins, 20 mM HEPES, 0.1% BSA, pH 7.4). The cortex was isolated, minced on ice, and fragments of cortical tubules were dissociated by gentle pressure and filtration through two nylon filters of different pore sizes (first ø 150 mm, and then ø 100 mm). This procedure removed most of the glomeruli; the preparation contains up to 90% of proximal tubule fragments.

2.2. $^{86}Rb^+$ uptake

After incubation of cortical tubule cells at 37°C in the presence of the various agents (cAMP analogues, forskolin+IBMX, H-89, or ouabain) according to the protocol, the transport activity of Na^+,K^+-ATPase was determined as previously described [1]. Ouabain-sensitive $^{86}Rb^+$ uptake was calculated as the difference between the mean values measured in triplicate samples incubated with or without ouabain. $^{86}Rb^+$ uptake was expressed as pmol Rb^+ mg protein^{-1} min^{-1} ± S.E.M. and presented as means of relative uptake values with respect to the control values ± S.E.M. from n independent experiments.

2.3. Phosphorylation level evaluation

$^{32}P_i$ incorporation into Na^+,K^+-ATPase was estimated by laser-densitometry quantification of autoradiograms obtained after Na^+,K^+-ATPase immunoprecipitation from ^{32}P-labelled cortical tubule cells incubated in the presence of the various agents (cAMP analogues, forskolin+IBMX, H-89). Immunoprecipitation was performed with a rabbit polyclonal anti-Na^+,K^+-ATPase antibody raised against the purified rat kidney holoenzyme. Characterization of this antibody and the detailed experimental procedure to evaluate the phosphorylation level were described elsewhere [1]. Results are expressed as means of the relative intensity of each band with respect to the controls ± S.E.M. from n independent experiments. Usually, each experiment contained 2–3 control groups and their mean was taken as the control value.

2.4. Statistics

Statistical comparison of PKA activator-treated tubules versus corresponding untreated tubules was evaluated by Mann-Whitney U-test, with $p < 0.05$ considered significant. In time-course and dose-dependence experiments, the relationship between phosphorylation and cation transport activity was analyzed by linear regression.

3. Results and discussion

To examine the role of cAMP signalling pathway in the regulation of the Na^+,K^+-ATPase in rat cortical kidney cells, we increased the intracellular cAMP content either by addi-

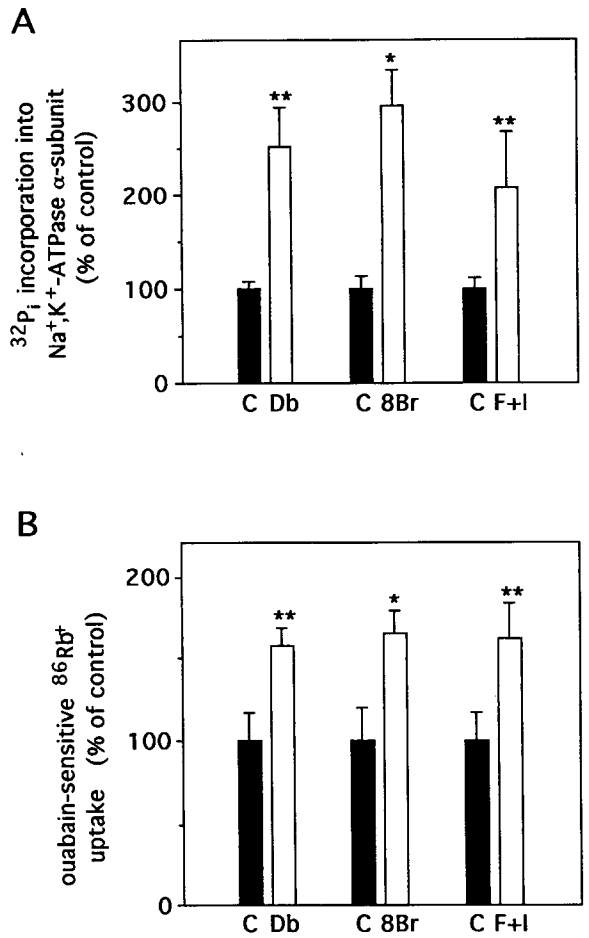

Fig. 2. Effects of PKA activators on ouabain-sensitive $^{86}Rb^+$ uptake and phosphorylation level of the Na^+,K^+-ATPase α-subunit. Tubule suspension was incubated in the absence (C, control) or presence of 1 mM Db-cAMP (Db), 1 mM 8-Br-cAMP (8Br) or with 0.01 mM forskolin plus 0.1 mM IBMX (F+I). (A) Quantification of relative intensities of $^{32}P_i$ incorporation into α-subunit. (B) Ouabain-sensitive $^{86}Rb^+$ uptake measured under initial rate. Data are expressed as a percentage of control values and are means ± S.E.M. from 4–11 independent experiments. $^*p < 0.05$ and $^{**}p < 0.01$.

tion of 8-bromo-cAMP or dibutyryl-cAMP, two cell-permeant analogues of cAMP, or by activation of the adenylyl cyclase by forskolin with simultaneous inhibition of cyclic nucleotide phosphodiesterases by 3-isobutyl-1-methylxanthine.

Cortical tubule suspensions of rat kidney labelled with [^{32}P]phosphate were incubated in the presence or absence of 1 mM 8-bromo-cAMP (8Br-cAMP), 1 mM dibutyryl-cAMP (Db-cAMP), or 0.01 mM forskolin plus 0.1 mM IBMX (F+I). After homogenization of the tubules, equal amounts of proteins were submitted to immunoprecipitation with a polyclonal anti-Na^+,K^+-ATPase antibody. Autoradiograms of the immunoprecipitation pattern displayed an enriched ^{32}P-labelled band (Fig. 1, lanes 1–6). Immunoblotting of membranes with the McK1 antibody identified this band as the α_1-subunit of the Na^+,K^+-ATPase (Fig. 1, lanes 7–8). Interestingly, under control conditions Na^+,K^+-ATPase α-subunit incorporated $^{32}P_i$ to a slight extent, suggesting that a turnover of phosphorylation of the enzyme occurs under basal conditions. Treatment of cortical suspension with the PKA activators increased the $^{32}P_i$ content of the α-subunit (Fig. 1, lanes 1–6). Plots of the relative intensities of $^{32}P_i$ incorporation into α-subunit are depicted in Fig. 2A and showed a 2–3-fold enhancement of $^{32}P_i$ incorporation into the Na^+,K^+-ATPase α-subunit under PKA activation conditions.

To examine the physiological relevance of these phosphorylation level changes, we measured the cation transport activity of Na^+,K^+-ATPase, by performing $^{86}Rb^+$ uptake experiments in the presence of the PKA activators. A 15 min incubation at 37°C of cortical tubule suspension in the presence of cAMP analogues (Db-cAMP or 8Br-cAMP) enhanced the ouabain-sensitive $^{86}Rb^+$ uptake by approx. 30% (Fig. 2B) without acting on ouabain-sensitive $^{86}Rb^+$ uptake (data not shown). Under the same conditions, forskolin plus IBMX also stimulated the ouabain-sensitive $^{86}Rb^+$ uptake (Fig. 2B). These results indicated that PKA activation led to the stimulation of Na^+,K^+-ATPase in rat cortical renal cells. This finding is in agreement with recent studies showing a stimulatory effect of cAMP on proximal Na^+,K^+-ATPase activity estimated by oxygen consumption [12], basolateral membrane potential variation [3] or Na^+,K^+-ATPase [γ-^{32}P]ATP hydrolytic activity [13]. The increases in ion transport activity and in the degree of phosphorylation of the Na^+,K^+-ATPase suggest that cAMP modulates the enzyme activity by promoting the phosphorylation of its α-subunit. In agreement with the present results, it has recently been demonstrated using a reconstituted enzyme preparation that PKA phosphorylation either does not affect or leads to an activation of Na^+,K^+-ATPase depending on the animal species origin of the enzyme preparation [11].

To assess whether PKA pathway activation is responsible for the observed effects of Db-cAMP, cortical tubule suspensions were incubated with H-89, an inhibitor of PKA [14]. 50 μM H-89 did not affect the basal phosphorylation level of the Na^+,K^+-ATPase or its basal transport activity, but prevented both the enhancement of α-subunit phosphorylation and the increase in $^{86}Rb^+$ uptake produced by Db-cAMP (Fig. 3).

Altogether, these results indicated that the increased Na^+,K^+-ATPase α-subunit phosphorylation and increased cation transport activity of the pump were induced, in part, by the specific activation of PKA. They also suggested that a kinase other than PKA is implicated in the tonic regulation of the basal phosphorylation as incubation of tubules in the presence of H-89 did not affect this basal phosphorylation level. In a previous work [1], we found that this basal phosphorylation was not blunted by GF109203X, an inhibitor of PKC. The possibility of measuring the catalytic phosphorylated intermediate is excluded by the conditions used for gel electrophoresis, the phosphate linked to the aspartyl residue being labile under alkaline conditions.

To confirm the role of PKA activation in the enhancement of α-subunit phosphorylation and of Na^+,K^+-ATPase activity, we performed time course and dose-dependence experiments. Time course effects on the Na^+,K^+-ATPase phosphorylation level and ouabain-sensitive $^{86}Rb^+$ uptake using 1 mM Db-cAMP are shown in Fig. 4B. For these experiments, the total incubation time was 30 min. 1 mM Db-cAMP was added at various times from 0 to 30 min. An increase in phosphorylation level induced by Db-cAMP was already apparent after 5 min of incubation and lasted over the 30 min period of incubation in the presence of Db-cAMP (Fig. 4A,B). Similar time course effects were observed in transport activity

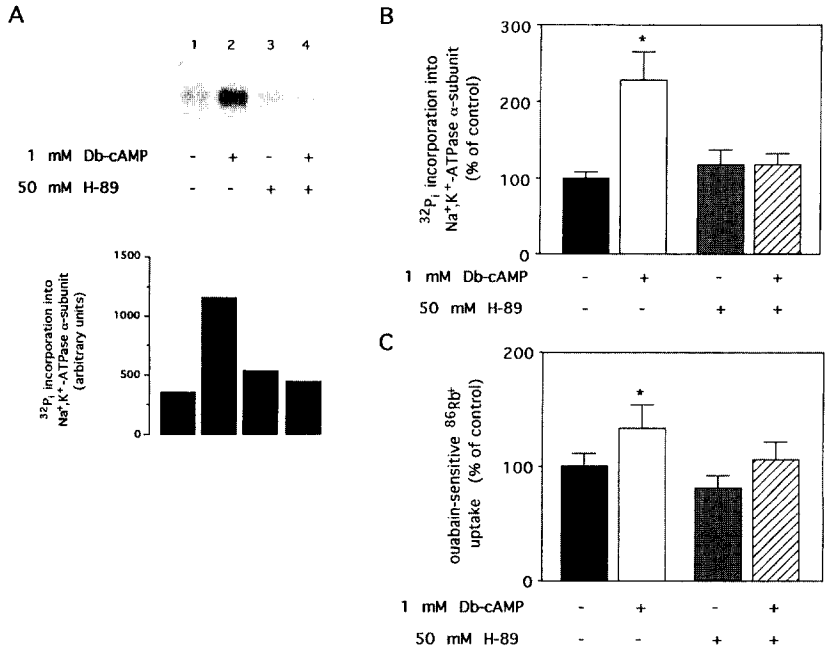

Fig. 3. Effects of PKA inhibition on ouabain-sensitive $^{86}Rb^+$ uptake and phosphorylation level of the Na^+,K^+-ATPase α-subunit. Tubule suspension was incubated 30 min at 37°C in the absence or presence of 1 mM Db-cAMP, 50 μM H-89 and 50 μM H-89 plus 1 mM Db-cAMP. Where appropriate, the presence of Db-cAMP was only for the last 15 min period of incubation. (A) (Upper panel) Representative autoradiogram of immunoprecipitated Na^+,K^+-ATPase. (Lower panel) Quantification of the $^{32}P_i$ incorporation into α-subunit shown in upper panel. (B) Quantification of relative intensities of $^{32}P_i$ incorporation into α-subunit. (C) Ouabain-sensitive $^{86}Rb^+$ uptake measured under initial rate. Data in B,C are expressed as a percentage of control values and are means ± S.E.M. from 6–9 independent experiments. $^*p < 0.05$.

experiments (Fig. 4B). No changes in ouabain-insensitive $^{86}Rb^+$ uptake were observed (data not shown). These results indicated that Db-cAMP exerts a parallel effect on the phosphorylation level and on the transport activity of Na^+,K^+-ATPase ($r^2 = 0.82$; Fig. 4C).

The dose dependence of the effects of Db-cAMP on Na^+,K^+-ATPase phosphorylation and $^{86}Rb^+$ uptake was investigated by incubating the tubule suspension with increasing concentrations of Db-cAMP. The results are depicted in Fig. 5. No changes in ouabain-sensitive $^{86}Rb^+$ uptake were observed (data not shown). Phosphorylation and $^{86}Rb^+$ uptake dose-response curves are correlated ($r^2 = 0.88$; Fig. 5C). The Db-cAMP-induced increase in phosphorylation of the α-subunit was observed at 0.1 mM Db-cAMP concentration (Fig. 5A,B), while the increase in Na^+,K^+-ATPase activity required a 10-fold higher concentration (Fig. 5B). These results suggest that a threshold level of phosphorylation has to be reached to stimulate the transport activity.

The present time- and dose-dependence experiments support the hypothesis of Na^+,K^+-ATPase regulation by phosphorylation. Our results demonstrated enhanced P_i incorporation into the α-subunit of Na^+,K^+-ATPase under specific PKA activation conditions. They are strengthened by the fact that several groups recently identified a single highly conserved PKA phosphorylation site on the α-subunit of Na^+,K^+-ATPase (Ser-943) [8,9]. Moreover, direct phosphorylation by PKA has been demonstrated in in vitro studies with purified [7] and reconstituted [11] Na^+,K^+-ATPase. However, in vivo direct α-subunit Na^+,K^+-ATPase phosphorylation by PKA remains to be established. Although the present results demonstrated the existence of a phosphorylation process through PKA in intact cells, they did not distinguish between a direct or indirect effect of PKA on Na^+,K^+-ATPase. It remains possible that PKA acts by regulating another protein kinase or a protein phosphatase located downstream in its pathway. There is an increasing number of recognized Ser/Thr phosphatases which are regulated by phosphorylation processes. For instance, PKA phosphorylates inhibitor-1 (I-1), a small protein known to inhibit type-1 protein phosphatases (PP1) [15]. Phosphorylation events have also been implicated in the regulatory mechanisms of the type-2 protein phosphatases (PP2): it has been proposed that direct phosphorylation by PKA of some regulatory subunit of type-2 protein phosphatase (PP2A) promotes a different set of substrates probably by influencing the interactions between the catalytic subunit and the different regulatory subunits. Thus, a model proposing inactivation of a protein phosphatase after phosphorylation by PKA could be considered.

We propose that phosphorylation changes the transport activity of the pump by promoting a spatial rearrangement of the enzyme, facilitating its interaction with substrates (Na^+,K^+, or Mg-ATP). Other likely mechanisms explaining an increasing in Na^+,K^+-ATPase activity in response to Db-cAMP include activation of quiescent enzymes present in the membrane or upregulation of already active pumps. Phosphorylation of the α-subunit could lead to enzyme stimulation by promoting the association of Na^+,K^+-ATPase with a cellular stimulating factor; or conversely, by promoting the dissociation of a cellular inhibitory factor from a preformed complex between Na^+,K^+-ATPase and this factor.

In conclusion, our data indicate that stimulation of cellular PKA provokes increased phosphorylation of the α-subunit of

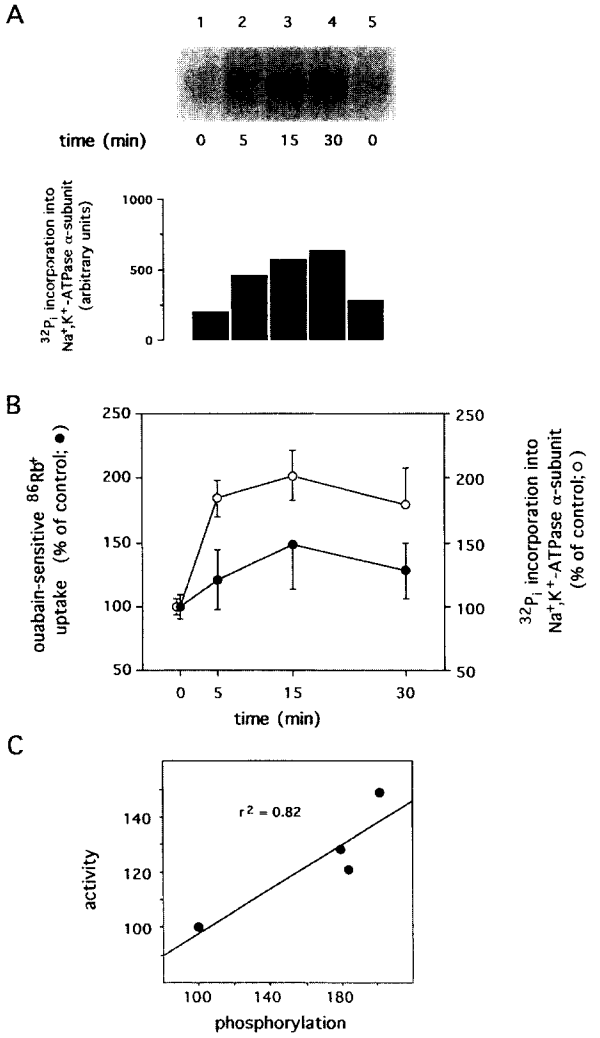

Fig. 4. Time course of Db-cAMP effect on ouabain-sensitive $^{86}Rb^+$ uptake and phosphorylation level of the Na^+,K^+-ATPase α-subunit. Tubule suspension was incubated in the presence of 1 mM Db-cAMP for 0, 5, 15, or 30 min at 37°C. Total incubation time was 30 min for all groups. (A) (Upper panel) Representative autoradiogram of immunoprecipitated Na^+,K^+-ATPase. (Lower panel) Quantification of $^{32}P_i$ incorporation into α-subunit shown in upper panel. (B) Effects of incubation time in the presence of 1 mM Db-cAMP on phosphorylation level (○) and transport activity (●). Data are expressed as a percentage of control values and are means ± S.E.M. from at least 5 independent experiments. (C) Linear regression analysis between increases in phosphorylation and ouabain-sensitive $^{86}Rb^+$ uptake.

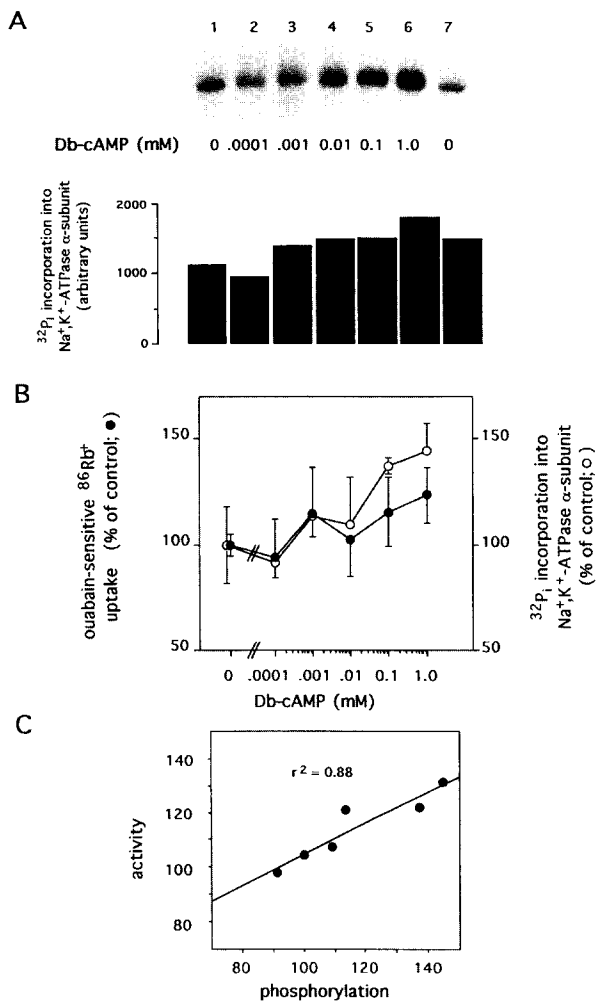

Fig. 5. Dose dependence of Db-cAMP effects on ouabain-sensitive $^{86}Rb^+$ uptake and phosphorylation level of the Na^+,K^+-ATPase α-subunit. Tubule suspension was incubated with increasing Db-cAMP concentration (10^{-7}–10^{-3} M). (A) (Upper panel) Representative autoradiogram of immunoprecipitated Na^+,K^+-ATPase. (Lower panel) Quantification of $^{32}P_i$ incorporation into α-subunit shown in upper panel. (B) Effects of increasing Db-cAMP concentrations on phosphorylation level (○) and transport activity (●). Data are expressed in percentage of control values and are means ± S.E.M. from 2–6 independent experiments. (C) Linear regression analysis between increases in phosphorylation and ouabain-sensitive $^{86}Rb^+$ uptake.

Na^+,K^+-ATPase recovered in homogenates of rat cortical kidney as well as increased $^{86}Rb^+$ uptake by intact cells of rat cortical kidney. They provide evidence that Na^+,K^+-ATPase is a physiological substrate for protein kinases in vivo. The definition of the proteins involved in phosphorylation/dephosphorylation of α-subunit Na^+,K^+-ATPase will allow a better understanding of the means by which hormones regulate the enzyme activity and thus how they control the sodium reabsorption by kidney.

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