

Protein kinase A induces recruitment of active Na⁺,K⁺-ATPase units to the plasma membrane of rat proximal convoluted tubule cells

Maria Luisa Carranza, Martine Rousselot, Alexander V. Chibalin*,
Alejandro M. Bertorello*, Hervé Favre and Eric Féraille

*Laboratoire de Néphrologie, Fondation pour Recherches Médicales, Avenue de la Roseraie 64, CH-1211 Genève 4, Switzerland and *Department of Molecular Medicine, Karolinska Institute, Rolf Luft Centre for Diabetes Research, Karolinska Hospital, 17176 Stockholm, Sweden*

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1. The aim of this study was to investigate the mechanism of control of Na⁺,K⁺-ATPase activity by the cAMP–protein kinase A (PKA) pathway in rat proximal convoluted tubules. For this purpose, we studied the *in vitro* action of exogenous cAMP (10⁻³ M dibutyryl-cAMP (db-cAMP) or 8-bromo-cAMP) and endogenous cAMP (direct activation of adenylyl cyclases by 10⁻⁵ M forskolin) on Na⁺,K⁺-ATPase activity and membrane trafficking.
2. PKA activation stimulated both the cation transport and hydrolytic activity of Na⁺,K⁺-ATPase by about 40%. Transport activity stimulation was specific to the PKA signalling pathway since (1) db-cAMP stimulated the ouabain-sensitive ⁸⁶Rb⁺ uptake in a time- and dose-dependent fashion; (2) this effect was abolished by addition of H-89 or Rp-cAMPS, two structurally different PKA inhibitors; and (3) this stimulation was not affected by inhibition of protein kinase C (PKC) by GF109203X. The stimulatory effect of db-cAMP on the hydrolytic activity of Na⁺,K⁺-ATPase was accounted for by an increased maximal ATPase rate (*V*_{max}) without alteration of the efficiency of the pump, suggesting that cAMP–PKA pathway was implicated in membrane redistribution control.
3. To test this hypothesis, we used two different approaches: (1) cell surface protein biotinylation and (2) subcellular fractionation. Both approaches confirmed that the cAMP–PKA pathway was implicated in membrane trafficking regulation. The stimulation of Na⁺,K⁺-ATPase activity by db-cAMP was associated with an increase (+40%) in Na⁺,K⁺-ATPase units expressed at the cell surface which was assessed by Western blotting after streptavidin precipitation of biotinylated cell surface proteins. Subcellular fractionation confirmed the increased expression in pump units at the cell surface which was accompanied by a decrease (–30%) in pump units located in the subcellular fraction corresponding to early endosomes.
4. In conclusion, PKA stimulates Na⁺,K⁺-ATPase activity, at least in part, by increasing the number of Na⁺–K⁺ pumps in the plasma membrane in proximal convoluted tubule cells.

In the kidney proximal convoluted tubule (PCT), the fluid and solute reabsorption rate is controlled by hormones and neurotransmitters which possess receptors coupled to adenylyl cyclase and cAMP-dependent protein kinase (PKA) (for review see Morel & Doucet, 1986). The effects of cAMP on the tubular reabsorption processes are mediated by regulation of the activities of ion transporters expressed in both apical and basolateral membrane domains. Sodium apical entry occurs mainly through the amiloride-sensitive Na⁺–H⁺ exchanger (Preisig & Rector, 1988) and accessorially through Na⁺-coupled solute cotransporters (Lapointe *et al.*

1986). In addition, the recent discovery of an apical Na⁺ channel (Willmann *et al.* 1997) raises the possibility of a second amiloride-sensitive Na⁺ entry pathway. Basolaterally, Na⁺ exits the cell primarily through Na⁺,K⁺-ATPase and subsidiarily through the Na⁺–HCO₃⁻ cotransporter (Ullrich *et al.* 1977; Yoshitomi *et al.* 1985). By extruding sodium ions from the renal cells, Na⁺,K⁺-ATPase provides the driving force for the vectorial reabsorption processes (Ullrich *et al.* 1977).

In PCT, short-term stimulation of Na⁺,K⁺-ATPase by conditions that activate PKA has been reported by several

groups (Giesen *et al.* 1984; Bertorello & Aperia, 1988; Breton *et al.* 1994; Beck *et al.* 1995; Carranza *et al.* 1996) but the mechanisms have yet to be determined. Besides the possibility of an increase in Na^+, K^+ -ATPase activity due to the kinetic effect of a rise in intracellular Na^+ concentration (Skou, 1965), a change in the intrinsic properties of Na^+, K^+ -ATPase (Férraille *et al.* 1995) or an increased number of active $\text{Na}^+ - \text{K}^+$ pump units expressed in plasma membranes (Hundal *et al.* 1992) may explain the effect of PKA activation.

In this study, we have investigated how the cAMP-PKA signalling pathway controls Na^+, K^+ -ATPase activity in rat PCT. We found that PKA activation stimulated Na^+, K^+ -ATPase activity, at least in part, by increasing the number of $\text{Na}^+ - \text{K}^+$ pumps in the plasma membrane.

METHODS

Isolation of single pieces of nephron

Male Wistar rats (130–150 g; Physiologie Institute, Bern, Switzerland) were anaesthetized with pentobarbitone (5 mg (100 g body weight)⁻¹ i.p.) and the left kidney was quickly perfused through the abdominal aorta with 4 ml of incubation solution (see composition below) containing 0.18% (w/v) collagenase (Serva, CLSII, 0.87 U mg⁻¹). In order to reduce renal anoxia, the aorta was ligated above the left renal artery junction just before starting the perfusion. After perfusion, the left kidney was immediately removed and the rat killed by exsanguination. The kidney was sliced into small pyramids which were incubated at 30 °C for 20 min in aerated incubation solution containing 0.05% (w/v) collagenase. The pyramids were then thoroughly rinsed in ice-cold incubation solution and stored in ice until use. The tubules were microdissected under stereomicroscopic control in oxygenated incubation solution (Férraille *et al.* 1995) containing 120 mM NaCl, 5 mM RbCl, 4 mM NaHCO₃, 1 mM CaCl₂, 1 mM MgSO₄, 0.2 mM NaH₂PO₄, 0.15 mM Na₂HPO₄, 5 mM glucose, 10 mM lactate, 1 mM pyruvate, 4 mM glutamine, 4 mM essential and non-essential amino acids (Gibco), 0.03 mM vitamins (Gibco), 0.1% (w/v) bovine serum albumin (BSA) and 20 mM Hepes, pH 7.45.

⁸⁶Rb⁺ uptake

The transport activity of Na^+, K^+ -ATPase was measured in intact isolated tubules by ouabain-sensitive ⁸⁶Rb⁺ uptake, as previously described (Cheval & Doucet, 1990). Prior to the ⁸⁶Rb⁺ uptake assay, tubules were pre-incubated at 37 °C for various times in incubation solution either alone or containing PKA activators and/or inhibitors and/or 5 mM ouabain.

Ouabain-sensitive ⁸⁶Rb⁺ uptake was calculated as the difference between the mean values measured in samples incubated in the presence and absence of ouabain. ⁸⁶Rb⁺ uptake values are expressed as picomoles of ⁸⁶Rb⁺ per millimetre of tubule per minute.

Na^+, K^+ -ATPase activity

The hydrolytic activity of Na^+, K^+ -ATPase was determined, as previously described (Doucet *et al.* 1979), by a radiochemical assay based on the measurement of ³²P_i (inorganic phosphate) released from [γ -³²P]ATP after permeabilization of cell membranes. Prior to the ATPase assay, the samples were pre-incubated at 37 °C for 15 min in ATPase assay solution either alone or containing 10⁻³ M db-cAMP.

The total ATPase activity assay solution contained 100 mM NaCl, 50 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 100 mM Tris-HCl, 10 mM MgATP, and trace amounts (5 nCi μl^{-1}) of [γ -³²P]ATP (Dupont de Nemours, Boston, 2–10 Ci mmol⁻¹). The pH of the solution was adjusted to 7.4. For Na^+ -independent ATPase activity measurements, NaCl was replaced by 150 mM KCl.

For each condition, ATPase activity was determined in five to seven replicates. Na^+, K^+ -ATPase activity was taken as the difference between the mean activities of total ATPase and Na^+ -independent ATPase. Na^+, K^+ -ATPase activity was taken as the Na^+ -dependent ATPase activity in order to avoid measurement of the ouabain-sensitive, K^+ -dependent and Na^+ -independent K^+ -ATPase activity recently described in rat PCT (Younes-Ibrahim *et al.* 1995). Results are expressed as picomoles of ATP per millimetre of tubule per minute.

Biotinylation of cell surface proteins

Glomeruli-free cortical tubule suspensions (containing ~90% proximal tubules) were prepared from collagenase-treated kidneys, as previously described (Carranza *et al.* 1996). Isolated tubules were incubated at 37 °C for 15 min in BSA-free incubation solution either alone or containing 10⁻³ M db-cAMP. Incubation was stopped on ice and tubules were pelleted by centrifugation. The supernatant was discarded and tubules were resuspended in biotinylation buffer solution (10 mM Tris-HCl (pH 7.5), 2 mM CaCl₂, 50 mM NaCl) containing 1.5 mg ml⁻¹ biotin (EZ-Link sulphosuccinimidobiotin (sulfo-NHS-biotin), Pierce). The biotinylation reaction was performed at 4 °C for 1 h with gentle end-over-end motion to ensure mixing. Tubules were then rinsed once with phosphate-buffered saline (PBS)-calcium-magnesium (CM) (composition (mM): 137 NaCl, 2.7 KCl, 8 Na₂PO₄, 1.5 KH₂PO₄, 0.1 CaCl₂ and 1 MgCl₂, pH 7.4) supplied with 100 mM glycine and then washed in this buffer solution for 20 min at 4 °C to quench all of the free biotin. After one further rinse with PBS-CM, the tubules were lysed in 200 μl of 1% (w/v) sodium deoxycholate. After protein content determination of each sample, equal amounts of protein (30 μg) were added to 100 μl of streptavidin-agarose beads (Immunopure immobilized streptavidin, Pierce), diluted in an antiprotease-containing buffer solution (composition: 50 mM Tris-HCl (pH 7.4), 1 M NaCl, 5 mM EDTA, 10 μg ml⁻¹ aprotinin, 50 μg ml⁻¹ leupeptin), and 0.5% (w/v) digitonin. To ensure equal conditions, volume was adjusted with 1% (w/v) sodium deoxycholate. Samples were incubated overnight at 4 °C with end-over-end motion. The beads were then washed once with rinsing solution A (composition: 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.5% digitonin), twice with rinsing solution B (composition: 500 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.05% digitonin), three times with rinsing solution C (composition: 500 mM NaCl, 20 mM Tris-HCl (pH 7.4), 0.2% BSA, 0.25% digitonin) and once with 10 mM Tris-HCl (pH 7.4). After this last wash, the samples were resuspended in 100 μl of Laemmli's sample buffer solution (Laemmli, 1970) and submitted to SDS-PAGE. After electrotransfer of proteins from polyacrylamide gels, polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore) were incubated overnight at 4 °C with McK1 (Felsenfeld & Sweadner, 1988), a monoclonal anti- Na^+, K^+ -ATPase α 1-subunit antibody (courtesy of Dr K. J. Sweadner, Massachusetts General Hospital, Boston, MA, USA) diluted at 1:200 (v/v) or with monoclonal anti-Hsp27 antibody (StressGen) diluted 1:100 in Tris-buffered saline (TBS)-Tween (composition: 150 mM NaCl, 50 mM Tris, 0.2% Tween 20, pH 7.5) containing 1% BSA. The immunoreactivity was detected by the chemiluminescence method (ECL), according to the manufacturer's instructions (Amersham).

Each Western blot had its own internal calibration curve. This calibration curve consisted of loading serial dilutions of total proteins on each gel and thus allowed us to check the accuracy of the signal obtained with experimental samples.

Subcellular fractionation

Cortical tubules (containing ~90% PCT cells) from rat kidneys were prepared as previously described (Bertorello, 1992). After removal of the kidneys, the cortices were isolated and the tissue was minced on ice to a paste-like consistency. The preparation was incubated with $7.5 \text{ mg (100 ml collagenase)}^{-1}$ (type I, Sigma) for 60 min at 37°C in 50 ml oxygenated incubation solution (see 'Isolation of single pieces of nephron'). Incubation was stopped by placing the tissue on ice and pouring it through graded sieves (180, 75, 53 and $38 \mu\text{m}$ in pore size) to obtain a cell suspension. The cells were collected by centrifugation at $100 g$ for 4 min and washed three times in ice-cold incubation solution and resuspended. Cell suspensions were then incubated in the presence or absence of 10^{-3} M db-cAMP for 15 min at 37°C and then incubation was stopped by placing the cell suspension on ice.

Endosomes were fractionated on a flotation gradient as recently described (Chibalin *et al.* 1997). Briefly, after centrifugation and removal of the incubation solution, proximal tubule cells were suspended in ice-cold homogenization buffer solution containing 3 mM imidazole (pH 7.4), 250 mM sucrose, 2 mM EGTA, 10 mM NaF, 30 mM $\text{Na}_4\text{O}_7\text{P}_2$, 1 mM Na_3VO_4 , 1 mM PMSF as well as $10 \mu\text{g ml}^{-1}$ of leupeptin and $10 \mu\text{g ml}^{-1}$ of aprotinin. Cells were gently homogenized by fifteen to twenty strokes in a Dounce homogenizer. The homogenate was centrifuged at $3000 g$ for 5 min at 4°C . The post-nuclear supernatant was adjusted to 40.6% sucrose and loaded (1.5 ml) at the bottom of a 5 ml centrifuge tube, to which were added sequentially 16% sucrose (1.5 ml) in 3 mM imidazole and 0.5 ml EDTA; 10% sucrose in the same buffer (1 ml); and finally homogenization buffer (1 ml). The samples were centrifuged at $110000 g$ for 1 h at 4°C . Early endosomes were collected at the homogenization buffer–10% sucrose interface, and the late endosomes at the 10%–16% sucrose interface. The identity of early and late endosomes was confirmed by the presence of Rab5, a small GTPase that is localized on early endosomes and which controls early endosome fusion, and mannose-6-phosphate receptor, respectively. Basolateral plasma membranes were prepared from the fraction collected at the 16%–40.6% sucrose interface using a Percoll gradient. The collected material was diluted in homogenization buffer (see above) and centrifuged at $20000 g$ for 20 min at 4°C . The yellow layer was resuspended in the

supernatant and centrifuged at $48000 g$ for 30 min at 4°C . The supernatant was discarded and the pellet resuspended in 1 ml buffer containing 300 mM mannitol and 12 mM Hepes (pH 7.6). To form a Percoll gradient, 0.19 g undiluted Percoll (Pharmacia Biotech) was added to the 1 ml suspension. The suspension was gently mixed and centrifuged at $48000 g$ for 30 min and the ring of basolateral membranes was collected.

Proteins from basolateral membrane, early and late endosome fractions were analysed by SDS-PAGE (7.5–15%) using Laemmli's buffer system, and identification of the α -subunit of $\text{Na}^+\text{,K}^+$ -ATPase was performed using a monoclonal antibody raised against the $\alpha 1$ -subunit (Pietrini *et al.* 1992) (courtesy of Dr M. J. Caplan, Yale University, New Haven, CT, USA).

Statistics

The data are given as means \pm s.e.m. from n independent experiments. Each independent experiment was performed with one kidney from one rat. Statistical comparisons between two groups were performed by Student's t test for unpaired data. P values less than 0.05 were considered significant.

RESULTS

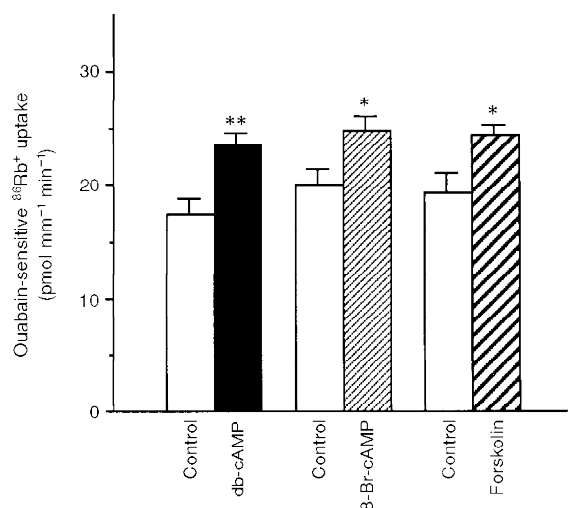
Effect of PKA activation on ouabain-sensitive $^{86}\text{Rb}^+$ uptake and $\text{Na}^+\text{,K}^+$ -ATPase activity

To examine the role of the cAMP signalling pathway in the regulation of $\text{Na}^+\text{,K}^+$ -ATPase in rat proximal convoluted tubules, we increased the intracellular cAMP content by addition of dibutyryl-cAMP (db-cAMP) or 8-bromo-cAMP (8-Br-cAMP), two cell-permeant analogues of cAMP, or by direct activation of adenylyl cyclases by forskolin.

An increase in the cellular levels of cAMP stimulated the cation transport activity of $\text{Na}^+\text{,K}^+$ -ATPase measured in intact rat PCTs in the presence of a rate-limiting intracellular Na^+ concentration (Cheval & Doucet, 1990). As shown in Fig. 1, a 15 min preincubation at 37°C of PCTs in the presence of either 10^{-3} M db-cAMP or 10^{-3} M 8-Br-cAMP stimulated ouabain-sensitive $^{86}\text{Rb}^+$ uptake by approximately 40% without acting on ouabain-insensitive $^{86}\text{Rb}^+$ uptake (data not shown). Under the same conditions, 10^{-5} M forskolin also stimulated ouabain-sensitive $^{86}\text{Rb}^+$ uptake by about 32% and did not alter ouabain-insensitive $^{86}\text{Rb}^+$

Figure 1. Activation of the cAMP-dependent protein kinase pathway stimulates ouabain-sensitive $^{86}\text{Rb}^+$ uptake in rat PCT

Ouabain-sensitive $^{86}\text{Rb}^+$ uptake was measured under initial rate conditions in PCTs preincubated for 15 min at 37°C in the absence (Control) or presence of one of the following PKA activators: 10^{-3} M db-cAMP, 10^{-3} M 8-Br-cAMP, or 10^{-5} M forskolin. Results are means \pm s.e.m. from 4–7 independent experiments. * $P < 0.05$, ** $P < 0.01$.



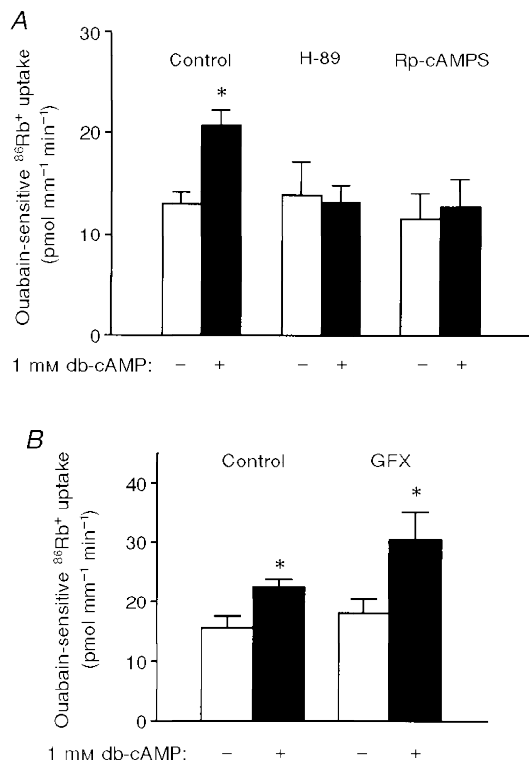


Figure 2. The effects of db-cAMP on ouabain-sensitive $^{86}\text{Rb}^+$ uptake are prevented when PKA but not PKC is inhibited

Ouabain-sensitive $^{86}\text{Rb}^+$ uptake was measured as described in Fig. 1. * $P < 0.05$. Results are means \pm s.e.m. from 4–5 independent experiments. *A*, PCTs were preincubated for 30 min at 37 °C in the absence (Control) or presence of either PKA inhibitor (5×10^{-5} M H-89 or 10^{-3} M Rp-cAMPS), and in the presence (+) or absence (-) of 10^{-3} M db-cAMP. *B*, PCTs were preincubated for 30 min at 37 °C in the absence (Control) or presence of a PKC inhibitor (5×10^{-7} M GF109203X, GFX), and in the presence (+) or absence (-) of 10^{-3} M db-cAMP.

uptake (data not shown). Since all PKA activators produced similar effects, db-cAMP was used in subsequent experiments.

To assess whether this stimulatory effect resulted from the activation of PKA, PCTs were incubated in the presence of two structurally different PKA inhibitors, *N*-(2-((*p*-bromo-

cinnamyl)amino)ethyl)-5-isoquinolinesulphonamide HCl (H-89) and *R_p*-cyclic 3',5'-hydrogen phosphorothiolate adenosine triethylammonium salt (Rp-cAMPS). As shown in Fig. 2*A*, preincubation of samples for 30 min at 37 °C in the presence of PKA inhibitors (5×10^{-5} M H-89 or 10^{-3} M Rp-cAMPS) did not significantly alter basal ouabain-sensitive $^{86}\text{Rb}^+$ uptake but prevented the stimulatory effect

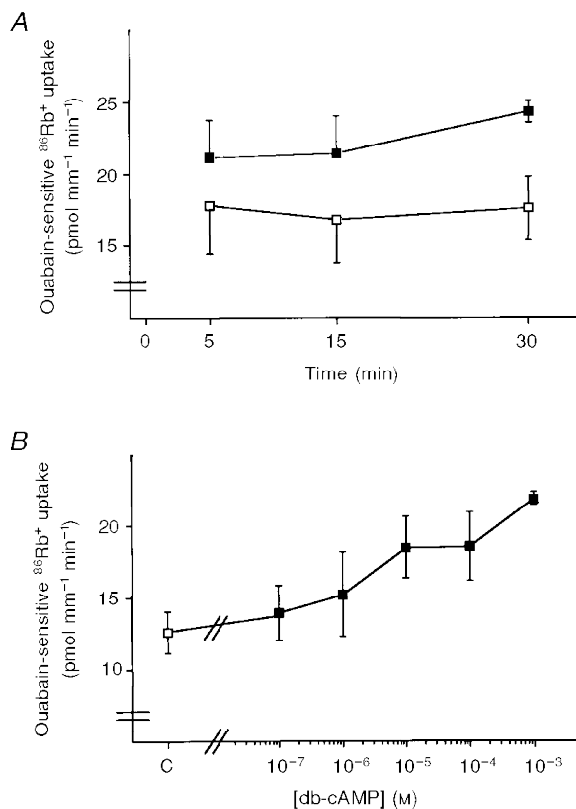


Figure 3. The effects of db-cAMP on ouabain-sensitive $^{86}\text{Rb}^+$ are time and dose dependent

Ouabain-sensitive $^{86}\text{Rb}^+$ uptake was measured as described in Fig. 1. *A*, PCTs were preincubated for 5, 15 or 30 min at 37 °C in the absence (□) or presence (■) of 10^{-3} M db-cAMP. Results are means and s.e.m. from 3 independent experiments. *B*, PCTs were preincubated for 30 min at 37 °C in the absence (□) or presence (■) of db-cAMP at various concentrations from 10^{-7} to 10^{-3} M. Results are means \pm s.e.m. from 6 independent experiments.

of db-cAMP on ouabain-sensitive ⁸⁶Rb⁺ uptake. By contrast, preincubation of the tubules in the presence of 5 × 10⁻⁷ M GF109203X (GFX), a specific PKC inhibitor, did not alter basal ouabain-sensitive ⁸⁶Rb⁺ uptake but appeared to increase the stimulatory effect of db-cAMP on ouabain-sensitive ⁸⁶Rb⁺ uptake (Fig. 2*B*). However, the difference between the db-cAMP-treated group and the GFX plus db-cAMP group was not significant (*P* = 0.17). H-89, Rp-cAMPS and GF109203X had no effect on ouabain-insensitive ⁸⁶Rb⁺ uptake (data not shown).

In order to better characterize the db-cAMP effects on Na⁺,K⁺-ATPase, we performed time and dose dependence experiments. The time course of the db-cAMP (10⁻³ M) effect on ouabain-sensitive ⁸⁶Rb⁺ uptake is shown in Fig. 3*A*. Stimulation of ouabain-sensitive ⁸⁶Rb⁺ uptake by db-cAMP was already apparent after 5 min of preincubation at 37 °C and reached a plateau after 15 min. The effect of db-cAMP on ouabain-sensitive ⁸⁶Rb⁺ uptake was concentration dependent with a threshold effect observed at 10⁻⁵ M (Fig. 3*B*).

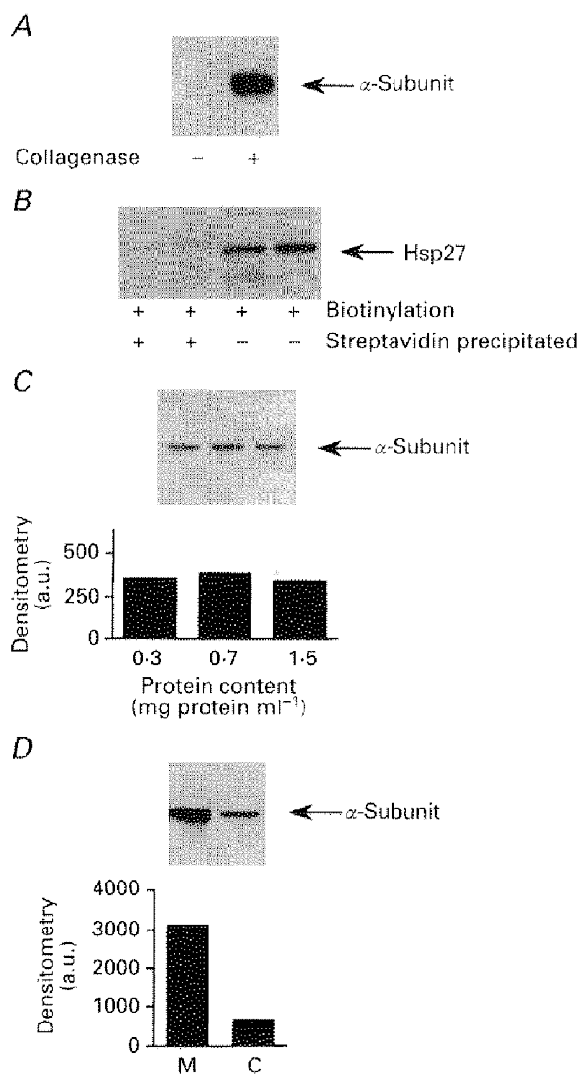
The stimulatory effects of db-cAMP on ouabain-sensitive ⁸⁶Rb⁺ uptake could be mediated either by modulating the

intracellular Na⁺ concentration through an increase in apical entry or by acting directly on the pump. Thus we investigated the effect of db-cAMP on the hydrolytic activity of Na⁺,K⁺-ATPase in the absence of a Na⁺ gradient. In PCTs preincubated for 15 min in the presence of 10⁻³ M db-cAMP, Na⁺,K⁺-ATPase activity measured in the presence of a saturating concentration of sodium (100 mM) was stimulated by about 45% (in pmol ATP mm⁻¹ min⁻¹: control, 48.07 ± 6.0; db-cAMP, 69.79 ± 5.0, *P* > 0.05), indicating that db-cAMP increased the *V*_{max} of Na⁺,K⁺-ATPase. Na⁺-independent ATPase activity was not altered by db-cAMP (data not shown). This increase represents either a direct effect on maximal enzyme turnover or an increase in the number of active Na⁺,K⁺-ATPase units.

To evaluate the possibility of an additional effect of db-cAMP on the Na⁺ affinity of Na⁺,K⁺-ATPase and/or on intracellular Na⁺ concentration (secondary to an alteration in its apical entry), we calculated the efficiency of Na⁺,K⁺-ATPase for each experimental condition (control or db-cAMP) as the ratio of the mean of corresponding ⁸⁶Rb⁺ uptake values and the mean of Na⁺,K⁺-ATPase activity values, and assuming a stoichiometry of two Rb⁺ ions transported for each hydrolysed ATP molecule. In agreement

Figure 4. Determination of experimental conditions for the biotinylation assay

After biotinylation and lysis of cortical tubules, equal amounts of total protein were submitted to streptavidin precipitation. The Na⁺,K⁺-ATPase α1-subunit was detected with McK1, a monoclonal antibody raised against Na⁺,K⁺-ATPase α1-subunit. *A*, effect of removal of the basal membrane on Na⁺,K⁺-ATPase accessibility. Cortical tubules were obtained from untreated (left lane) or collagenase-treated (right lane) kidneys. *B*, absence of intracellular protein biotinylation. Hsp27, a cytosolic protein, was detected by immunoblot before (right 2 lanes) and after (left 2 lanes) streptavidin precipitation of biotinylated samples. *C*, biotinylation of cell surface protein is performed under saturating conditions. Increasing amounts of cortical tubules were incubated in the presence of 1.5 mg ml⁻¹ biotin and equal amounts of total protein extracts were submitted to streptavidin precipitation. The bottom panel represents densitometry results of the upper blot. *D*, suspensions of tubules obtained from the kidney medulla (M) or cortex (C) were biotinylated and equal amounts of total proteins were submitted to streptavidin precipitation. The bottom panel represents densitometry results of the upper blot.



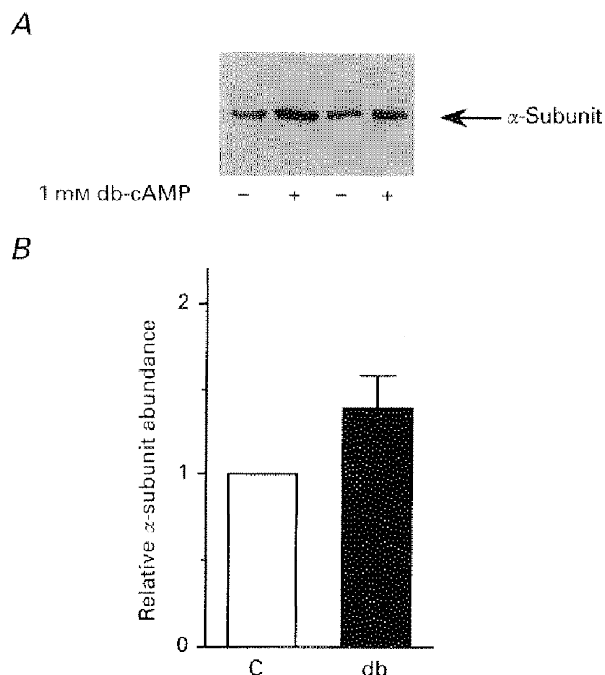


Figure 5. The number of Na^+ , K^+ -ATPase units is increased at the cell surface after db-cAMP treatment in rat cortical tubules

Cortical tubules ($\sim 90\%$ PCTs) isolated from collagenase-treated kidneys were preincubated for 15 min at 37°C in the absence (C, control) or presence of 10^{-3} M db-cAMP (db) before biotinylation for 1 h with 1.5 mg ml^{-1} sulfo-NHS-biotin at 4°C . Tubules were then lysed with 1% sodium deoxycholate and equal amounts of total proteins ($30\ \mu\text{g}$) were precipitated on streptavidin beads. At least 2 samples were precipitated for each group of lysates. Proteins were separated by SDS-PAGE and transferred to PVDF membranes before Western blotting with the McK1 antibody. *A*, representative immunoblot from 9 independent experiments. *B*, scanning results from 9 independent experiments. Results are given as means \pm s.e.m. of the relative intensities; db-cAMP increased the cell surface expression of Na^+ , K^+ -ATPase by $41 \pm 19\%$ ($P < 0.05$).

with previous results (Cheval & Doucet, 1990), we found that in intact PCT cells, i.e. under intracellular Na^+ rate-limiting conditions, Na^+ , K^+ -ATPase functioned at 16% of its maximal capacity under basal conditions. The respective calculated efficiency for db-cAMP treated groups was similar (15%), strongly suggesting that db-cAMP did not significantly alter the Na^+ affinity of Na^+ , K^+ -ATPase and that its effect is independent of Na^+ availability.

Effects of db-cAMP on the cell surface expression of Na^+ , K^+ -ATPase

To assess whether or not db-cAMP modified the cell surface expression of Na^+ , K^+ -ATPase, we used the biotinylation–streptavidin precipitation procedure. To adapt this technique to our system we first determined the accessibility of

Na^+ , K^+ -ATPase to the biotin derivative. In renal epithelial cells, Na^+ , K^+ -ATPase is located in the basolateral plasma membrane to which the tubular basal membrane can prohibit the access of the sulfo-NHS-biotin. Since the Na^+ , K^+ -ATPase α -subunit was detected only in collagenase-treated tubules (Fig. 4*A*), digestion of tubular basal membrane with collagenase was used for all subsequent experiments.

A second important prerequisite for this procedure was the impermeability of the plasma membrane to sulfo-NHS-biotin. As shown in Fig. 4*B*, Hsp27, an abundant cytosolic protein, was detected by Western blotting (monoclonal antibody from StressGen) in total cellular extracts but not in the streptavidin precipitated samples, indicating the impermeability of the plasma membrane to sulfo-NHS-biotin.

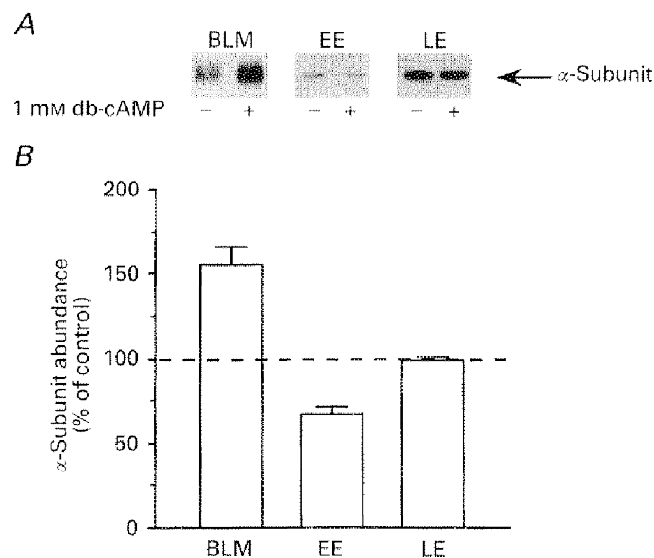


Figure 6. Increase in Na^+ , K^+ -ATPase units in the plasma membrane after db-cAMP treatment is associated with a decrease in pump units in early endosomes in rat cortical tubule cells

Cortical tubule cell suspensions ($\sim 90\%$ PCT cells) were preincubated for 15 min at 37°C in the absence or presence of 10^{-3} M db-cAMP before subcellular fractionation. Equal amounts of protein from both control and db-treated samples were loaded onto SDS-PAGE. The loaded amounts for each fraction were $5\ \mu\text{g}$ of protein from basolateral membrane (BLM), and $1\ \mu\text{g}$ from early (EE) and late endosomes (LE). *A*, representative immunoblots of the α -subunit of Na^+ , K^+ -ATPase with an anti- $\alpha 1$ -subunit monoclonal antibody. *B*, scanning results from 3 independent experiments. Data are expressed as a percentage of control values and are means \pm s.e.m.; db-cAMP increased the amount of α -subunit by $55 \pm 10\%$ ($P < 0.05$) in BLM, decreased the amount of α -subunit by $33 \pm 4\%$ ($P < 0.05$) in EE and did not alter the α -subunit content of LE ($-2 \pm 3\%$).

We further checked whether the biotinylation procedure was performed under saturating conditions. Increasing amounts of cortical tubules (from 0.3 to 1.5 mg total protein ml^{-1}) were incubated in the presence of the same amount of sulfo-NHS-biotin. After cell lysis, equal amounts of total protein extracts (30 μg) were incubated with an excess of streptavidin-coupled agarose beads. As shown in Fig. 4C, similar amounts of $\text{Na}^+\text{-K}^+$ -ATPase α -subunits were recovered under each condition, indicating that maximal labelling of the plasma membrane $\text{Na}^+\text{-K}^+$ pumps was achieved.

Finally, the intensity of the $\text{Na}^+\text{-K}^+$ -ATPase α -subunit signal detected by immunoblot after streptavidin precipitation of biotinylated proteins should be proportional to the number of pump units expressed at the cell surface. This was confirmed by a threefold greater cell surface expression of $\text{Na}^+\text{-K}^+$ -ATPase in the inner stripe of the outer medulla than in the cortex (Fig. 4D), in agreement with the known differences in expression of the enzyme between these two parts of the kidney tissue (McDonough *et al.* 1994).

Based on these results, we assumed that the biotinylation–streptavidin precipitation procedure was appropriate for the purpose of the experiment. Thus, after incubation in the presence or absence of 10^{-3} M db-cAMP for 15 min at 37 °C, cortical tubules were subjected to the biotinylation–streptavidin precipitation procedure. Western blotting analysis showed a 40% increase in $\text{Na}^+\text{-K}^+$ -ATPase α -subunit immunoreactivity in db-cAMP-treated samples compared with control samples (Fig. 5), indicating that db-cAMP increased the cell surface expression of $\text{Na}^+\text{-K}^+$ -ATPase.

Effects of db-cAMP on $\text{Na}^+\text{-K}^+$ -ATPase distribution in cellular membrane compartments

In order to confirm that db-cAMP increased the number of $\text{Na}^+\text{-K}^+$ -ATPase units present in the plasma membrane, we performed subcellular fractionation experiments. After incubation of cortical tubules in the presence or absence of 10^{-3} M db-cAMP, the $\text{Na}^+\text{-K}^+$ -ATPase α -subunit was detected by Western blotting in plasma membrane, early or late endosome fractions. As shown in Fig. 6, db-cAMP induced a net increase in α -subunit abundance in the plasma membrane fraction that was proportional to the reciprocal net loss observed in the early endosome fraction, while no changes were found in the late endosome fraction. These results suggest that db-cAMP induces a redistribution of $\text{Na}^+\text{-K}^+$ -ATPase between an intracellular compartment and the plasma membrane.

DISCUSSION

The results of this study indicate that the stimulatory effects of the cAMP–PKA pathway on $\text{Na}^+\text{-K}^+$ -ATPase activity are mainly due to an increase in the number of $\text{Na}^+\text{-K}^+$ -ATPase units present in the plasma membrane of the proximal tubule. The effect of db-cAMP on the $^{86}\text{Rb}^+$ uptake and the change in the number of pump units

expressed at the cell surface were of the same order of magnitude (~30–40%) under conditions of rate limiting Na^+ concentration.

Our results are in agreement with studies demonstrating a stimulatory effect of cAMP on proximal tubule $\text{Na}^+\text{-K}^+$ -ATPase activity measured in intact cells by (a) ouabain-sensitive oxygen consumption (Beck *et al.* 1995), (b) $\text{Na}^+\text{-K}^+$ -ATPase-dependent basolateral membrane potential variation (Breton *et al.* 1994), (c) ouabain-sensitive $^{86}\text{Rb}^+$ uptake (Carranza *et al.* 1996), or in permeabilized isolated PCTs (Bertorello & Aperia, 1988, 1989) and kidney cortex homogenate (Giesen *et al.* 1984) by the hydrolytic activity of the enzyme. However, one study has reported inhibition of $\text{Na}^+\text{-K}^+$ -ATPase by db-cAMP (Ribeiro & Mandel, 1992) while another one did not find any effect of either exogenous or endogenous cAMP on $\text{Na}^+\text{-K}^+$ -ATPase activity (Satoh *et al.* 1993). These discrepancies are neither explained by species differences nor by specific experimental conditions, such as temperature, oxygenation status, and bicarbonate or calcium concentrations in the incubation solutions.

In the kidney proximal convoluted tubule, solute and water reabsorptions are under the control of hormones and neurotransmitters possessing receptors coupled to adenylate cyclase and cAMP-dependent protein kinase (PKA) which can exert contradictory effects according to the ion transport system considered. For example in microperfused proximal tubule, cAMP decreases bicarbonate (McKinney & Myers, 1980) and increases chloride (Wang *et al.* 1995) reabsorption. These contradictory effects of cAMP depend on the regulation of ion transporters expressed in both apical and basolateral membrane domains. The inhibitory action of cAMP on bicarbonate reabsorption may result from a coordinated inhibition of the apical $\text{Na}^+\text{-H}^+$ exchanger (Weinman, Shenolikar & Kahn, 1987) and of the basolateral $\text{Na}^+\text{-HCO}_3^-$ cotransporter (Ruiz & Arruda, 1992). Therefore, the stimulatory effect of cAMP on chloride reabsorption requires both apical and basolateral Cl^- transport that is independent of acid–base transport; this is most likely to be via cAMP-regulated apical (Darvish *et al.* 1994) and basolateral Cl^- channels (Seki *et al.* 1995). The basolateral Cl^- extrusion is favoured by the $\text{Na}^+\text{-K}^+$ -ATPase-dependent hyperpolarization that follows exposure of cells to cAMP (Breton *et al.* 1994). In addition, the stimulation of $\text{Na}^+\text{-K}^+$ -ATPase can also increase the driving force for paracellular Cl^- reabsorption. Furthermore, the sustained stimulation of $\text{Na}^+\text{-K}^+$ -ATPase transport activity by cAMP implies an adequate Na^+ entry independent of the $\text{Na}^+\text{-H}^+$ exchanger. The organic solutes– Na^+ -coupled cotransporters and the described apical amiloride-sensitive Na^+ conductance (Willmann *et al.* 1997) are potential candidates. In the PCT this Na^+ conductance could be created by epithelial Na^+ (ENa) channels similar to those described in the collecting duct (Willmann *et al.* 1997). These channels might be activated when Na^+ entry through the apical $\text{Na}^+\text{-H}^+$ exchanger is decreased. Indeed, ENa channel activity is closely related to Na^+ availability and is upregulated by a

decreased rate of Na^+ entry (Frindt *et al.* 1993; Willmann *et al.* 1997). Conversely, ENa channel activity is down-regulated when the rate of the Na^+ entry exceeds the rate of the Na^+ exit driven by the Na^+, K^+ -ATPase activity (Silver *et al.* 1993). Furthermore, cAMP increases the amiloride-sensitive Na^+ conductance of the apical membrane (Schafer & Troutman, 1990) through direct stimulation of ENa channels in the collecting duct (Frindt & Palmer, 1996). Thus, one may postulate that in PCT, ENa channel activity regulation is similar to that observed in the collecting duct. Taken together, it is fair to postulate that cAMP promotes a shift from sodium bicarbonate towards sodium chloride reabsorption in PCT. In this context, the stimulation of Na^+, K^+ -ATPase by cAMP could play a key role in the enhanced NaCl reabsorption by maintaining or increasing the electrochemical gradients necessary to the sodium and chloride reabsorptions independently of the apical $\text{Na}^+ - \text{H}^+$ exchanger and basolateral $\text{Na}^+ - \text{HCO}_3^-$ cotransporter activities.

Our results show that activation of PKA increases the V_{max} of Na^+, K^+ -ATPase as well as the number of plasma membrane pump units in rat PCTs. Thus an increase in enzyme turnover or activation of latent pump units already present in plasma membranes does not contribute to the increase in V_{max} . In addition, the rapid time course of the db-cAMP effect on the pump activity makes the synthesis of new pump units unlikely. Therefore, the observed proportional redistribution of $\text{Na}^+ - \text{K}^+$ pumps between plasma membrane and early endosomes can fully explain the cAMP-induced increase in Na^+, K^+ -ATPase activity. This result suggests that latent intracellular $\text{Na}^+ - \text{K}^+$ pumps located in an early endosomal compartment can be translocated in response to PKA activation in PCT cells. However, a PKA-induced decrease in the endocytosis rate of Na^+, K^+ -ATPase cannot be excluded.

Since the cAMP pathway evokes activation of at least one protein kinase, one may hypothesize that phosphorylation processes are implicated in the redistribution of $\text{Na}^+ - \text{K}^+$ pumps. Na^+, K^+ -ATPase α -subunit phosphorylation has been shown to occur *in vitro* (Bertorello *et al.* 1991; Chibalin *et al.* 1992) and in intact cells (Beguín *et al.* 1994; Fisone *et al.* 1994; Carranza *et al.* 1996). We have recently shown that an increase in the phosphorylation status of Na^+, K^+ -ATPase occurs concomitantly with ouabain-sensitive $^{86}\text{Rb}^+$ uptake stimulation by db-cAMP in intact cells of rat kidney cortex (Carranza *et al.* 1996). However, the definite relationship between this cAMP-stimulated phosphorylation and the effect of the PKA pathway on Na^+, K^+ -ATPase activity remains to be determined. It would be a valuable endeavour to test whether Na^+, K^+ -ATPase α -subunit phosphorylation by PKA regulates the distribution of $\text{Na}^+ - \text{K}^+$ pumps between plasma membrane and intracellular compartments.

In conclusion, our data indicate that activation of cellular PKA by cAMP stimulates Na^+, K^+ -ATPase activity in rat proximal tubules. This effect is probably mediated by the

insertion of additional Na^+, K^+ -ATPase units in the plasma membrane, implying that the cAMP-PKA pathway contributes in the control of membrane trafficking.

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Corresponding author

M. L. Carranza: Laboratoire de Néphrologie, Fondation pour Recherches Médicales, Avenue de la Roseraie 64, CH-1211 Genève 4, Switzerland.

Email: carranza@cmu.unige.ch