Effect of insulin on Na^+,K^+-ATPase in rat collecting duct

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1. The collecting duct is involved in the whole antinatriuretic effect of insulin, as indicated *in vitro* by the stimulatory effect of the hormone on ouabain-sensitive $^{86}Rb^+$ uptake. Since Na^+,K^+-ATPase drives Na^+ reabsorption, the contribution of the Na^+ pump to the effect of insulin was investigated in rat isolated cortical and outer medullary collecting duct.

2. Insulin enhanced ouabain-sensitive $^{86}Rb^+$ uptake in the absence, as well as in the presence, of either 5×10^{-4} M amiloride or 10^{-3} M hydrochlorothiazide (HCT). Maximal ouabain-sensitive $^{86}Rb^+$ uptake, measured in Na^+-loaded tubules, was also enhanced by insulin. The insulin effect persisted both in the absence of external Na^+, when the Na^+,K^+-ATPase operates in a Rb^+–Rb^+ exchange mode, and in tubules depolarized by a high external concentration (20 mM) of Rb^+ or by addition of 3 mM Ba^{2+}.

3. Insulin treatment did not alter the intracellular Na and K concentrations, the specific binding of [^{3}H]ouabain measured in intact tubules, or the hydrolytic activity of Na^+,K^+-ATPase measured after permeabilization of the tubule cells.

4. In conclusion, in the rat collecting duct, insulin increased Na^+,K^+-ATPase-mediated cation transport independently of Na^+ availability, membrane potential and recruitment of pump units. The effect of insulin was lost after cell permeabilization, suggesting the presence of a cytosolic factor which controls the turnover of Na^+,K^+-ATPase.

In healthy humans (De Fronzo, Cooke, Andres, Faloona & Davis, 1975) and in various animal species (De Fronzo, Goldberg & Agus, 1976; Kirchner, 1988), insulin exerts an antinatriuretic effect independently of glycaemic status. The direct action of insulin on the kidney is demonstrated by the observation that insulin decreases Na^+ excretion both in isolated perfused kidneys (Nizet, Lefebvre & Crabbé, 1971) and in the ipsilateral kidney of dogs during intrarenal insulin infusion (De Fronzo *et al.* 1976). From the isolated perfused kidney preparation, it appears that the net salt-retaining effect of insulin results from the preponderance of its stimulatory effect on tubular Na^+ reabsorption over its ability to increase the glomerular filtration rate (Cohen, McCarthy & Stoff, 1989). Direct control of tubular Na^+ reabsorption by insulin has been further demonstrated in isolated microperfused tubules (Baum, 1987; Furuya, Tabei, Muto & Asano, 1992; Mandon, Siga, Chabardès, Firsov, Roinel & de Rouffignac, 1993).

Among the different segments of the nephron, the collecting duct is of major importance as the site of the final control of the urinary Na^+ excretion. In the isolated rat collecting duct, we have reported that physiological concentrations of insulin enhance the rate of Na^+,K^+-ATPase-dependent cation transport, as measured by the ouabain-sensitive moiety of rubidium uptake (Féraille *et al.* 1992) which estimates the transepithelial rate of Na^+ transport. Our results were in agreement with previous reports demonstrating that insulin stimulated transepithelial Na^+ transport in toad bladder (Cobb, Yang, Brown & Scott, 1986) and A6 cells (Fidelman, May, Biber & Watlington, 1982), models of mammalian collecting duct and principal cells, respectively. These findings suggest that insulin physiologically controls the Na^+ reabsorption taking place in the collecting duct.

However, the mechanism of the action of insulin on tubular Na^+ transport has not been directly studied in the collecting duct. Whether the effect of insulin results from a direct stimulation of Na^+,K^+-ATPase or is mediated by an increase in Na^+ entry and its subsequent rise in intracellular concentration has not been established. Therefore, the present study was designed to elucidate the mechanism of the stimulation of the Na^+,K^+-ATPase-mediated cation transport by insulin in the rat collecting duct. For this purpose, we measured the effect of insulin on (i) ouabain-sensitive $^{86}Rb^+$ uptake; (ii) intracellular concentration of Na; (iii) specific [^{3}H]ouabain binding; and (iv) hydrolytic activity of Na^+,K^+-ATPase.

METHODS

Animals and isolation of single pieces of nephron

Experiments were carried out on male Wistar rats (Physiologie Institute, Bern, Switzerland) weighing 130–150 g and fed standard laboratory chow. After anaesthesia (5 mg (100 g body wt)$^{-1}$ sodium pentobarbitone I.P.) the left kidney was quickly perfused through the abdominal aorta with 4 ml of solution A (for composition see Table 1) containing 0·18% (w/v) collagenase (0·87 U mg^{-1}; CLSII; Serva). In order to reduce renal anoxia, the aorta was ligated above the left renal artery junction just before starting the perfusion. After perfusion, the kidney was immediately removed and sliced into small pyramids which were incubated at 30 °C for 20 min in aerated incubation solution containing 0·05% (w/v) collagenase. Pyramids were then thoroughly rinsed in ice-cold incubation solution and stored at 0–4 °C until use. The tubules were microdissected under stereomicrocrospic control in different incubation solutions, according to the experimental design (see below and Table 1). The animals were killed by exsanguination.

Determination of $^{86}Rb^+$ uptake

$^{86}Rb^+$ uptake was determined on intact tubules, as previously described (Cheval & Doucet, 1990). For the experimental series designed to study the effect of amiloride and hydrochlorothiazide (HCT), microdissection was performed in solution A. Seven tubules were transferred in 1 μl of solution A to the concavity of a sunken bacteriological slide and preincubated for 15 min at 37 °C after the addition of another 1 μl of solution A (see Table 1) in the absence or presence of 2×10^{-8} M insulin, 5×10^{-3} M ouabain, 10^{-3} M amiloride or 2×10^{-3} M HCT, as indicated in legends to figures and tables. The uptake of $^{86}Rb^+$ began after addition of 0·5 μl of the solution used during the preincubation step containing trace amounts of ^{86}RbCl (100 nCi per sample; Amersham, UK) which was pre-equilibrated at 37 °C. Incubation was stopped after 1 min by adding 50 μl of ice-cold rinsing solution containing (mM): 150 choline chloride, 1·2 $MgSO_4$, 1·2 $CaCl_2$, 2 $BaCl_2$, 5 Hepes; osmolality was adjusted to 400 mosmol (kg H_2O)$^{-1}$ with mannitol; pH 7·40. Tubules were then rapidly washed in three successive baths of ice-cold rinsing solution, then individually transferred with 0·2 μl of the last rinsing bath onto a small microscope slide, photographed for determination of tubular length, and dropped into a counting vial containing 0·5 ml of 1% (w/v) deoxycholic acid. The radioactivity contained in each sample was determined by liquid scintillation. All values were corrected with the blank value determined from five to ten replicate samples consisting of 0·2 μl of the last rinsing bath. Ouabain-sensitive $^{86}Rb^+$ uptake was calculated as the difference between the mean values measured in samples incubated with and without ouabain (in (pequiv Rb^+) mm^{-1} min^{-1}).

Table 1. Composition of incubation solutions (mM)

	A	B	C	D
NaCl	120	120	0	105
RbCl	5	0	5	20
$CaCl_2$	1	1	1	1
$MgSO_4$	1	1	1	1
NaH_2PO_4	0·20	0·20	0	0·20
Na_2HPO_4	0·15	0·15	0	0·15
$NaHCO_3$	4	4	0	4
KH_2PO_4	0	0	0·20	0
K_2HPO_4	0	0	0·15	0
$KHCO_3$	0	0	4	0
Choline chloride	0	0	120	0
D-Glucose	5	5	5	5
Lactic acid	2	2	2	2
Amino acids	4	4	4	4
Vitamins	0·03	0·03	0·03	0·03
Hepes	20	20	20	20

The osmolality of incubation solutions was adjusted to 400 mosmol (kg H_2O)$^{-1}$ by addition of mannitol, and pH was adjusted to 7·40 with NaOH except for solution C in which KOH was used.

When the maximal rate of ouabain-sensitive $^{86}Rb^+$ uptake was determined, microdissection and preincubation steps were performed in solution B (K^+ and Rb^+ free, confirmed by flame photometry) to block Na^+,K^+-ATPase activity. This allows the intracellular Na^+ concentration to increase up to 100 mM (Blot-Chabaud, Jaisser, Gingold, Bonvalet & Farman, 1988). RbCl was then introduced together with $^{86}Rb^+$ to attain a final Rb^+ concentration of 5 mM which restored the Na^+,K^+-ATPase activity. $^{86}Rb^+$ uptake was stopped after 15 s.

When the cation transport activity of the Na^+,K^+-ATPase was determined under Rb^+–Rb^+ exchange mode conditions (Nokana, Warden & Stokes, 1992), microdissection, preincubation and $^{86}Rb^+$ uptake were performed in solution C (Na^+ free, confirmed by flame photometry).

For measurement of $^{86}Rb^+$ uptake in depolarized tubules, all experimental steps were performed either in solution D (which contained 20 mM RbCl) or in solution A supplemented with 3 mM $BaCl_2$.

Measurement of intracellular Na and K concentrations

Intracellular Na and K concentrations in the outer medullary collecting duct (OMCD) were measured by flame photometry as previously described (Sudo & Morel, 1984). Briefly, seven to ten tubules were transferred in 1 μl of solution A, in which KCl was substituted for RbCl in equimolar quantities, to the concavity of a sunken bacteriological slide. The tubules were incubated with or without 10^{-8} M insulin at 37 °C for 15 min. Incubation was stopped and tubules were rinsed according to the same procedure as for Rb^+ uptake measurement. After the last rinsing, tubules were individually photographed for determination of length and diameter, transferred with 50 nl of the last rinsing bath and treated for cation content measurement. In each experiment, Na and K concentrations were determined in five to seven replicates (mmol (l tubular volume)$^{-1}$).

Determination of [^{3}H]ouabain binding

[^{3}H]ouabain binding was measured in the intact cortical collecting duct (CCD) as previously described (El Mernissi & Doucet, 1984; Doucet & Barlet-Bas, 1989). After microdissection in the Rb^+- and K^+-free solution B, seven to ten tubules were transferred in 1 μl of solution B to the concavity of a sunken bacteriological slide. After addition of 1 μl binding medium (see composition below) in the absence or presence of 2×10^{-8} M insulin, tubules were incubated for 15 min at 37 °C, rinsed four times with ice-cold solution B and kept at 0–4 °C for 60 min. After two additional rinses, the tubules were transferred with 0·2 μl of the last rinsing bath into counting vials containing 0·5 ml of 1% (w/v) deoxycholic acid and radioactivity was counted by liquid scintillation.

The binding medium consisted of solution B (see Table 1) supplemented with 2 mM Na_3VO_4 and 1 mM [^{3}H]ouabain (10–20 Ci mmol^{-1}; Dupont de Nemours, Boston, MA, USA). Vanadate was used to block the pump in the E_2 configuration to which ouabain binds. Unlabelled ouabain (10 mM) together with KCl (60 mM) were added to measure non-specific binding. Specific [^{3}H]ouabain binding was calculated as the difference between the mean values of total and non-specific binding (in (fmol [^{3}H]ouabain) mm^{-1}).

Determination of the hydrolytic activity of Na^+,K^+-ATPase

The hydrolytic activity of Na^+,K^+-ATPase was determined by a radiochemical assay based on the measurement of inorganic phosphate released from γ-[^{32}P]ATP by the ATPase contained in a single segment of nephron as previously described (Doucet, Katz & Morel, 1979). Briefly, after a 15 min preincubation in solution A in the absence or presence of 10^{-8} M insulin, tubules were thoroughly rinsed with ice-cold distilled water and subjected to a freeze–thaw step in 0·2 μl of distilled water; this allowed removal of ions, permeabilization of cell membranes and the access of substrates into the cells. Then, 1 μl of ATPase assay solution (see composition below) was added to each sample. After incubation for 15 min at 37 °C, the reaction was stopped by cooling and addition of 5 μl 5% (w/v) cold trichloracetic acid. Samples were then transferred into 2 ml of 10% (w/v) activated charcoal. After mixing and centrifugation (2000 *g* for 10 min), the radioactivity of 500 μl aliquots of supernatant was measured by liquid scintillation.

The ATPase assay solution contained (mM): 10 $MgCl_2$, 1 EDTA, 100 Tris, 10 MgATP, 0–130 NaCl, 0–130 KCl, and trace amounts (5 nCi μl^{-1}) of γ-[^{32}P]ATP (2–10 Ci mmol^{-1}; Dupont de Nemours). The concentrations of Na^+ and K^+ were varied simultaneously to maintain the total $Na^+ + K^+$ concentration at 140 mM. The pH of the solutions was adjusted to 7·4.

Na^+,K^+-ATPase activity was taken as the difference between the total ATPase activity measured in the presence of both Na^+ and K^+ and, the Mg^{2+}-ATPase activity measured in the absence of Na^+ and in the presence of 5 mM ouabain. These activities were determined in five to seven replicates (in (pmol ATP) mm^{-1} min^{-1}).

Statistics and calculations

To assess the effect of insulin and inhibitors statistical comparisons between groups were performed using Student's *t* test for unpaired data, or when comparing several factors by analysis of variance. A *P* value of less than 0·05 was considered significant. Data are given as means ± S.E.M.

The Na^+ dependence of Na^+,K^+-ATPase was analysed using the Hill equation, assuming a cooperative model for binding of Na^+ ions as previously described (Féraille, Carranza, Rousselot & Favre, 1994):

$$v = V_{max}[Na^+]^{n_H}/([Na^+]^{n_H} + K_{0.5}{}^{n_H}),$$

and its linear expression:

$$\log\{v/(V_{max} - v)\} = n_H \log[Na^+] - n_H \log K_{0.5}, \quad (1)$$

where v is the Na^+,K^+-ATPase activity measured at $[Na^+]$, V_{max} is the maximal Na^+,K^+-ATPase activity, $K_{0.5}$ is the apparent dissociation constant for Na^+, and n_H is the Hill coefficient. Kinetic parameters of Na^+,K^+-ATPase were determined by non-linear regression or by linear regression analysis (Slide Write Plus 5.0; Advanced Graphics Software, Inc., Carlsbad, CA, USA).

RESULTS

All parameters, except the ATP hydrolytic activity of the Na^+ pump, were measured in intact living cells immediately at the end of the incubation period of tubules with insulin. Thus, (i) $^{86}Rb^+$ was simply added to the incubation medium containing insulin for 15 s or 1 min to measure Rb^+ uptake, (ii) [^{3}H]ouabain was added with insulin for measurement of the number of pump units present in the cell plasma membrane, and (iii) intracellular Na and K concentrations were measured after incubation with insulin. In contrast,

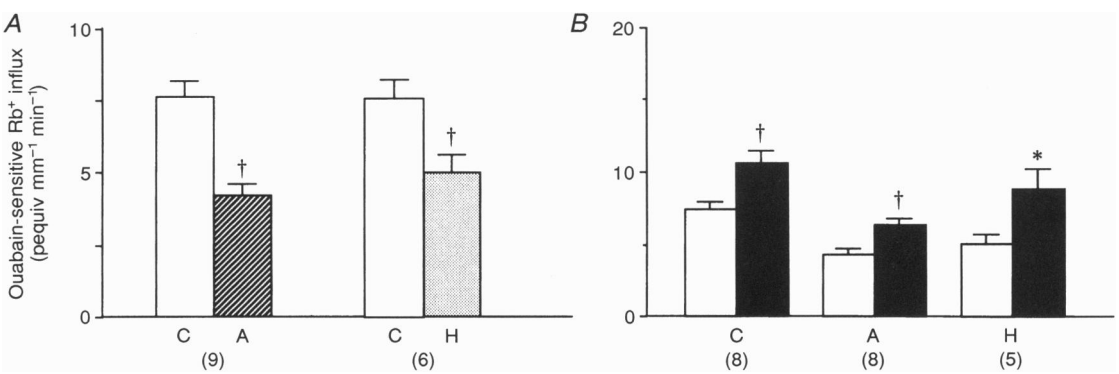

Figure 1. Effect of insulin on the initial rate of ouabain-sensitive $^{86}Rb^+$ influx in control, amiloride-treated and HCT-treated CCD

A, samples were microdissected and preincubated in solution A for 15 min at 37 °C either under control conditions (C; □) or in the presence of 5×10^{-4} M amiloride (A; ▨) or 10^{-3} M HCT (H; ▩) before measurement of $^{86}Rb^+$ uptake. *B*, samples were preincubated for 15 min at 37 °C in the absence (□) or presence (■) of 10^{-8} M insulin in the same mediums as in *A*. Results are means (S.E.M. indicated by bars) from several animals (number in parentheses) and statistical analysis was performed by two-way analysis of variance. Amiloride and HCT significantly decreased and insulin significantly enhanced ouabain-sensitive $^{86}Rb^+$ influx in the absence, as well as in the presence, of inhibitors (*$P < 0.05$; †$P < 0.01$).

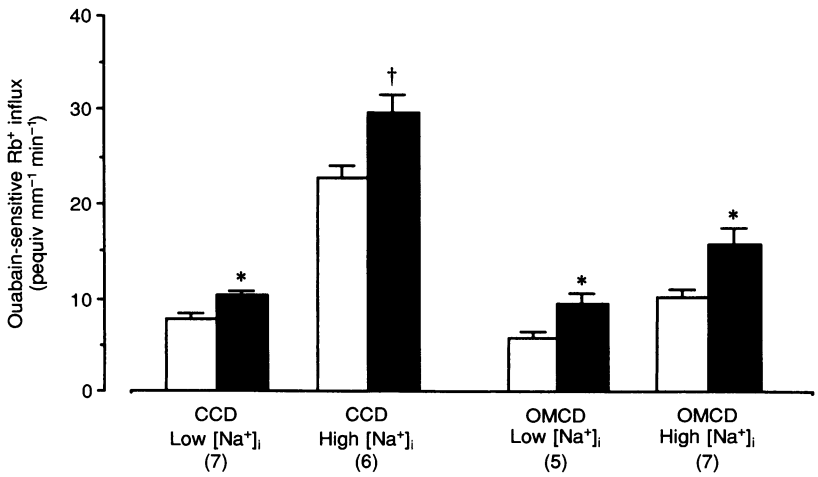

Figure 2. Effect of insulin on the initial rate of ouabain-sensitive $^{86}Rb^+$ uptake in unloaded or Na^+-loaded CCD and OMCD

Samples were preincubated for 15 min at 37 °C in the absence (□) or presence (■) of 10^{-8} M insulin, either in solution A (unloaded control tubules) or the K^+- and Rb^+-free solution B (Na^+-loaded tubules), before measurement of $^{86}Rb^+$ uptake. Statistical analysis was performed by two-way analysis of variance. Insulin significantly enhanced the ouabain-sensitive $^{86}Rb^+$ influx both in unloaded control or Na^+-loaded CCD and OMCD (*$P < 0.05$; †$P < 0.01$).

the ATP hydrolytic activity of the Na^+ pump was determined in tubule cells which had been permeabilized after the incubation step with insulin (see Methods).

Insulin action as studied by measurement of ouabain-sensitive $^{86}Rb^+$ uptake.

In the experiments presented below, insulin action was studied under appropriate conditions to analyse its possible modulation by intracellular Na^+ availability, membrane potential and/or Ba^{2+}-sensitive K^+ conductance.

Insulin action was studied in the absence and in the presence of 5×10^{-4} M amiloride or 10^{-3} M HCT in CCD, since the amiloride-inhibitable Na^+ conductive pathway and hydrochlorothiazide-inhibitable Na^+–Cl^- cotransport are the principal routes for Na^+ entry which support the Na^+ pump activity in the rat collecting duct (Terada & Knepper, 1990). The data presented in Fig. 1*A* show that amiloride and HCT significantly decreased the rate of ouabain-sensitive $^{86}Rb^+$ uptake from 7.6 ± 0.4 to 4.3 ± 0.4 pequiv mm^{-1} min^{-1} ($n = 9$; $P < 0.01$) and from 7.6 ± 0.6 to 5.1 ± 0.6 pequiv mm^{-1} min^{-1} ($n = 6$; $P < 0.05$), respectively, confirming the findings of Teruda & Knepper (1990). These effects probably reflect a decrease in intracellular Na^+ availability caused by the inhibition of Na^+ entry. As shown in Fig. 1*B*, an increase in ouabain-sensitive $^{86}Rb^+$ uptake was observed in response to insulin in control tubules (from 7.5 ± 0.5 to 10.5 ± 0.9 pequiv mm^{-1} min^{-1}; $n = 8$; $P < 0.01$) as well as in tubules treated with either amiloride (from 3.9 ± 0.3 to 6.1 ± 0.5 pequiv mm^{-1} min^{-1}; $n = 5$; $P < 0.01$) or HCT (from 5.6 ± 0.5 to 9.6 ± 1.5 pequiv mm^{-1} min^{-1}; $n = 5$; $P < 0.05$).

Insulin action was also studied in Na^+-loaded tubules (Fig. 2). Under these conditions in which the Na^+ pump operated under V_{max}, insulin increased the ouabain-sensitive $^{86}Rb^+$ uptake from 22.7 ± 1.4 to 29.7 ± 0.4 pequiv mm^{-1} min^{-1} ($P = 0.01$) and from 10.1 ± 0.8 to

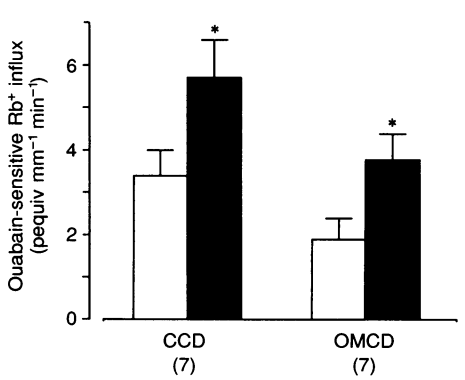

Figure 3. Effect of insulin on the initial rate of Na^+,K^+-ATPase-mediated Rb^+–Rb^+ exchange in CCD and OMCD

Samples were microdissected and preincubated for 15 min at 37 °C in solution C (without Na^+) in the absence (□) or presence (■) of 10^{-8} M insulin before measurement of $^{86}Rb^+$ uptake. Statistical analysis was performed by two-way analysis of variance. Insulin significantly enhanced the Na^+,K^+-ATPase-mediated Rb^+–Rb^+ exchange as measured by the ouabain-sensitive $^{86}Rb^+$ influx in both CCD and OMCD (*$P < 0.05$).

Table 2. Ouabain-insensitive $^{86}Rb^+$ uptakes (pequiv mm^{-1} min^{-1})

	CCD		OMCD	
	Control	Insulin	Control	Insulin
Solution A	2·5 ± 0·6	2·5 ± 0·4	2·4 ± 0·4	2·2 ± 0·4
Solution A + amiloride	3·1 ± 0·4	3·2 ± 0·4	—	—
Solution A + HCT	3·4 ± 0·4	3·6 ± 0·4	—	—
Solution B	6·5 ± 1·7	6·5 ± 1·2	6·9 ± 1·5	6·5 ± 1·4
Solution C	4·55 ± 0·6	4·61 ± 2·4	3·15 ± 0·8	3·9 ± 0·6
Solution D	14·3 ± 3·1	14·9 ± 6·4	—	—
Solution A + 3 mM $BaCl_2$	3·8 ± 0·8	4·3 ± 0·5	—	—

Results are means ± S.E.M. from the same animals as in Figs 1–4. Statistical analysis was performed by two-way analysis of variance. Under all the experimental conditions studied, insulin did not alter the ouabain-insensitive $^{86}Rb^+$ uptake.

15·6 ± 1·9 pequiv mm^{-1} min^{-1} ($P < 0·03$) in CCD ($n = 6$) and OMCD ($n = 7$), respectively. In unloaded tubules, the ouabain-sensitive $^{86}Rb^+$ uptake went from 7·7 ± 0·6 to 10·4 ± 0·4 pequiv mm^{-1} min^{-1} ($n = 7$; $P < 0·05$) and from 5·9 ± 0·6 to 9·4 ± 1·1 pequiv mm^{-1} min^{-1} ($n = 5$; $P < 0·025$) after insulin treatment for CCD and OMCD, respectively.

Insulin still induced an increase in ouabain-sensitive $^{86}Rb^+$ uptake when the Na^+ pump operated in Rb^+–Rb^+ exchange mode (Nonaka *et al.* 1992) in Na^+-depleted tubule cells (see Fig. 3) from 3·4 ± 0·6 to 5·7 ± 0·9 pequiv mm^{-1} min^{-1} and from 1·9 ± 0·5 to 3·8 ± 0·6 pequiv mm^{-1} min^{-1} in CCD and OMCD, respectively ($n = 7$; $P = 0·04$).

Altogether, the above data indicate that the increase in the ouabain-sensitive moiety of $^{86}Rb^+$ uptake by insulin (Féraille *et al.* 1992) occurred over a wide range of intracellular Na^+ concentrations.

In the following set of experiments, we examined the possible modulation of the insulin action by membrane potential and by Ba^{2+}-sensitive K^+ conductivity of the cell plasma membrane. It has been reported that Na^+ pump cation transport is voltage sensitive (Gadsby & Nakao, 1989; Palmer, Antonian & Frindt, 1993) and is coupled to K^+ conductive permeability pathways (Kone, Brady & Gullans, 1989; Tsuchiya, Wang, Giebisch & Welling, 1992). As depicted in Fig. 4, insulin still increased the ouabain-sensitive $^{86}Rb^+$ uptake from 11·1 ± 2·5 to 15·8 ± 2·7 pequiv mm^{-1} min^{-1} ($n = 5$; $P < 0·05$) when the membrane potential was depolarized by 20 mM extracellular RbCl. An increase from 4·9 ± 0·6 to 7·4 ± 1·1 pequiv mm^{-1} min^{-1} ($n = 7$; $P < 0·05$) was also observed when K^+ channels were blocked with 3 mM $BaCl_2$.

In contrast, insulin had no effect on the ouabain-insensitive moiety of $^{86}Rb^+$ uptake (Table 2) under all the experimental conditions studied. Taken together, these observations strongly suggest that insulin primarily increases the cation transport activity of the Na^+ pump. This insulin action can be induced and can operate independently of Na^+ availability, membrane potential and Ba^{2+}-sensitive K^+ conductivity.

Effect of insulin on intracellular Na and K concentrations

Cation concentrations were determined in OMCD incubated in solution A in which Rb^+ was substituted for K^+. Therefore, the experiments were performed under the same conditions as the experiments depicted in Fig. 1, i.e. with normal external concentrations of Na^+ (140 mM) and K^+ (5 mM) in the absence or presence of insulin (10^{-8} M). OMCD was chosen because it is easier to dissect and more convenient

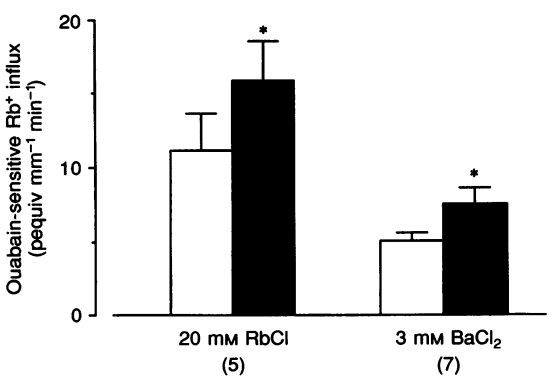

Figure 4. Effect of insulin on the initial rate of ouabain-sensitive $^{86}Rb^+$ uptake in depolarized CCD

Samples were preincubated for 15 min at 37 °C in solution D (containing 20 mM Rb^+) or in solution A supplemented with 3 mM $BaCl_2$ in the absence (□) or presence (■) of 10^{-8} M insulin before measurement of $^{86}Rb^+$ uptake. Statistical analysis was performed by two-way analysis of variance. Insulin significantly enhanced the ouabain-sensitive $^{86}Rb^+$ uptake both in the presence of 20 mM external Rb^+ or 3 mM $BaCl_2$ (*$P < 0·05$).

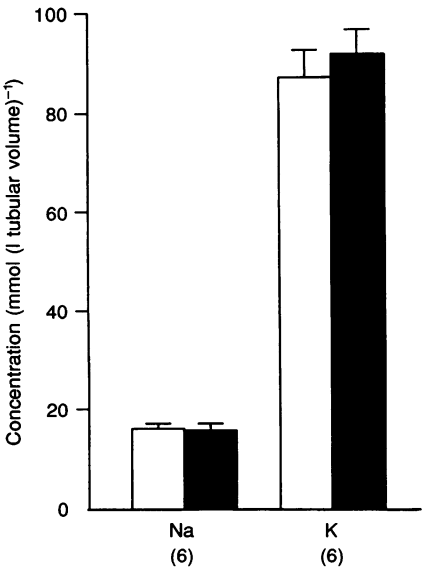

Figure 5. Intracellular Na and K concentrations in control and insulin-treated OMCD

Samples were preincubated for 15 min in the absence (□) or presence (■) of 10^{-8} M insulin before measuring intracellular Na and K concentrations by flame photometry. Na and K concentrations in insulin-treated tubules were not statistically different from controls using Student's t test for unpaired data.

than CCD when a large amount of material is required. As depicted in Fig. 5, under these conditions the mean intracellular Na concentrations were $16{\cdot}4 \pm 0{\cdot}7$ and $16{\cdot}0 \pm 1{\cdot}1$ mmol (l tubular volume)$^{-1}$ in control and insulin-treated tubules ($n = 6$), respectively. The corresponding intracellular K concentrations were $87{\cdot}5 \pm 5{\cdot}1$ (control) and $91{\cdot}7 \pm 5{\cdot}0$ mmol (l tubular volume)$^{-1}$ (insulin treated).

These results demonstrate that the insulin stimulation of the cation transport activity of the Na^+ pump (shown in Fig. 1) is not associated with any variation of the intracellular monovalent cation concentrations.

Effect of insulin on specific [^{3}H]ouabain binding

The capacity of insulin to enhance the maximal cation transport activity of the Na^+ pump (Fig. 2) indicates that insulin increases the number of active pump units in the cell plasma membrane, the turnover of each pump unit or both. Therefore, we measured the number of pump units present at the cell surface by the specific [^{3}H]ouabain binding. This measurement was performed in the absence of external K^+ and Rb^+, in Na^+-loaded tubules. As depicted in Fig. 6, there was no difference between the capacities of control and insulin-treated CCD to bind [^{3}H]ouabain: $9{\cdot}5 \pm 1{\cdot}2$ and $9{\cdot}3 \pm 1{\cdot}9$ fmol mm^{-1} for control and insulin-treated CCD, respectively ($n = 4$). Therefore, the increased cation transport capacity of the Na^+ pump shown in Fig. 2 does not result from an increase in the number of active pump units present in the cell plasma membrane.

Effect of insulin on the ATP hydrolytic activity of Na^+,K^+-ATPase

The ATP hydrolytic activity of Na^+,K^+-ATPase was measured in the presence of saturating concentrations of Na^+ (120 mM), and K^+ (10 mM). Under these V_{max} conditions, the pretreatment with insulin does not result in any change of the hydrolytic activity of Na^+,K^+-ATPase either in CCD, $18{\cdot}1 \pm 1{\cdot}5$ (control) and $17{\cdot}2 \pm 1{\cdot}1$ pmol mm^{-1} min^{-1} (insulin treated), or OMCD, $7{\cdot}4 \pm 1{\cdot}4$ (control) and $7{\cdot}5 \pm 1{\cdot}4$ pmol mm^{-1} min^{-1} (insulin treated) ($n = 5$). Mg^{2+}-ATPase activity was unchanged after insulin

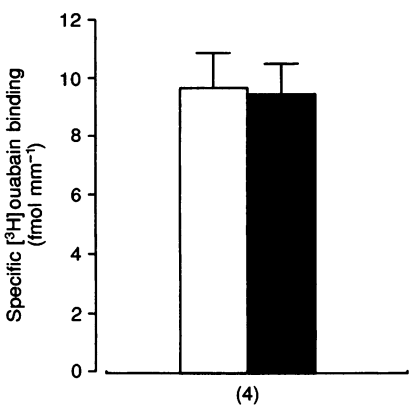

Figure 6. Specific [^{3}H]ouabain binding in control and insulin-treated CCD

For measurement of total ouabain binding, CCDs were incubated for 15 min at 37 °C in solution B (K^+ and Rb^+ free) supplemented with 1 mM vanadate and [^{3}H]ouabain concentrations up to 2×10^{-4} M in the absence (□) or presence (■) of 10^{-8} M insulin. Non-specific [^{3}H]ouabain binding was measured in the same medium containing 30 mM KCl and 5 mM unlabelled ouabain. Statistical analysis was performed using Student's t test for unpaired data. Specific [^{3}H]ouabain binding was similar in control and insulin-treated CCD.

treatment: for CCD, 15·3 ± 1·8 (control) and 15·9 ± 1·7 pmol mm^{-1} min^{-1} (insulin treated); for OMCD, 7·0 ± 1·3 (control) and 7·5 ± 1·0 pmol mm^{-1} min^{-1} (insulin treated).

When the Na^+ concentration of the assay medium was varied to investigate whether or not insulin alters the Na^+ sensitivity of the enzyme (Fig. 7), there was no difference between control and insulin-treated CCD in the estimated kinetic parameters, respectively: V_{max}, 16·2 ± 2·2 and 16·0 ± 2·1 mV; $K_{0.5}$ for Na^+, 23·0 ± 3·3 and 29·5 ± 4·3; n_H, 1·5 ± 0·2 and 1·5 ± 0·5.

Obviously, the data on the ATP hydrolytic activity of the collecting duct are in striking contrast with those on the cation transport activity given in Figs 1–4. This discrepancy is probably linked to the difference in the experimental procedures: $^{86}Rb^+$ uptake was measured in intact living tubule cells, whereas ATP hydrolytic activity was determined after cell permeabilization.

DISCUSSION

The collecting duct is critical for maintenance of the Na^+ homeostasis as the final adaptation of the Na^+ excretion by the kidney takes place in this segment of the nephron. This function of the collecting duct is predominantly dependent on hormonal control. The Na^+-regulating hormones, including insulin (De Fronzo *et al.* 1975), constitute the efferent pathway of the control system of the Na^+ homeostasis. For example, in the postprandial state, an insulin-mediated increase in Na^+ reabsorption by the collecting duct would be needed to prevent the Na^+ wastage expected from the increase in glomerular filtration rate characteristic of this physiological state. The collecting duct under these circumstances should rapidly increase its capacity of Na^+ reabsorption despite the low abundance of the Na^+,K^+-ATPase present in this segment. It is therefore interesting to investigate whether a specific mode of regulation of the enzyme operates in the collecting duct to face this challenge.

The present results establish that, as in the proximal convoluted tubule (PCT; Féraille *et al.* 1994) and in non-renal tissues (Lytton, 1985; Weil, Sasson & Gutman, 1991), Na^+,K^+-ATPase is directly regulated by insulin in rat collecting duct. Direct stimulation of the Na^+,K^+-ATPase activity could result from an increase in the number of Na^+ pump units and/or turnover rate of the enzyme. The insulin-induced increase in cation transport capacity of the collecting duct reported here is not associated with any

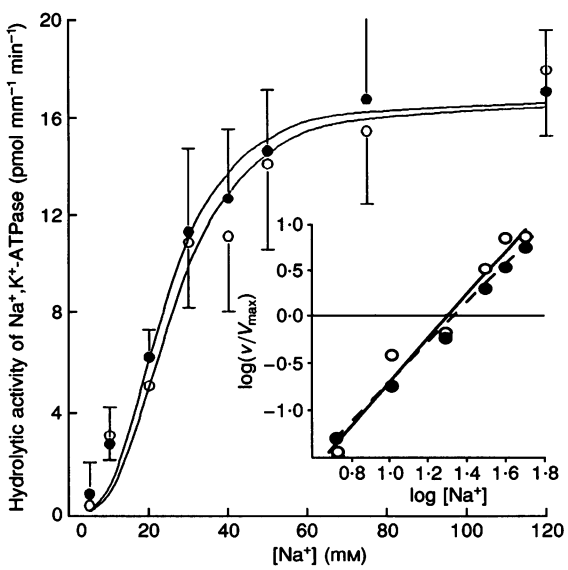

Figure 7. Na^+ dependence of the hydrolytic activity of Na^+,K^+-ATPase in control and insulin-treated permeabilized CCD

The tubules were preincubated in solution A for 15 min at 37 °C in the absence (○) or presence (●) of 10^{-8} M insulin. Na^+,K^+-ATPase activity was measured in the presence of concentrations of Na^+ ranging from 0 to 120 mM. The concentration of K^+ was adjusted to maintain the total $Na^+ + K^+$ concentration at 140 mM. The Na^+ activation curves of the Na^+,K^+-ATPase are the best fit of averaged data from 4 independent experiments with eqn (1) (control, $r^2 = 0·98$; insulin, $r^2 = 0·96$). The inset depicts the Hill plots from the same data (control, $r^2 = 0·97$; insulin, $r^2 = 0·99$). The V_{max}, taken as the Na^+,K^+-ATPase activity measured in the presence of 120 mM Na^+, was unchanged after insulin treatment (control, 18·0 pmol mm^{-1} min^{-1}; insulin, 17·1 pmol mm^{-1} min^{-1}). Also, insulin treatment did not change the $K_{0.5}$ of the enzyme for Na^+ (control, 26·8 mM; insulin, 22·8 mM) nor the Hill coefficient (control, 2·1; insulin, 2·0) estimated by linear regression analysis as described in Methods.

change in the number of Na^+ pump units as measured by specific [^{3}H]ouabain binding to the cell plasma membrane (Fig. 6). If one assumes that ouabain binding was not altered by the additional experimental step (storage at low temperature) technically mandatory (see Methods and Doucet & Barlet-Bas, 1989), the increased cation transport capacity by insulin-treated collecting ducts does not result from a recruitment of a latent pool of pump units into the cell plasma membrane (Barlet-Bas, Khadouri, Marsy & Doucet, 1990*b*; Blot-Chabaud, Wanstock, Bonvalet & Farman, 1990). Thus, this insulin effect has to be due to an increased turnover of each pump unit.

In rat, insulin directly stimulates Na^+,K^+-ATPase in the collecting duct as well as the proximal tubule; however, the mechanism of activation of the pump observed in the collecting duct differs from that operating in the proximal tubule. In the proximal tubule, insulin induced an increase in the Na^+ sensitivity of the enzyme without altering its V_{max} (Féraille *et al.* 1994); in the collecting duct, under the same experimental conditions, insulin increased the V_{max} of the pump without changing its Na^+ affinity. These differences in the activation mechanisms of the pump are not unique for insulin. In the proximal tubule, protein kinase C enhanced the Na^+ affinity of Na^+,K^+-ATPase (Féraille, Carranza, Buffin-Meyer, Rousselot, Doucet & Favre, 1995*b*); in the collecting duct, an increased V_{max} of the pump has been observed in rats with puromycin-induced nephrotic syndrome (Féraille *et al.* 1993). This apparent segment-specific regulation suggests either the presence of different isoforms of Na^+,K^+-ATPase or a specific modification of the intrinsic properties of the pump in the proximal tubule compared with the collecting duct. In the rabbit nephron, two putative isoforms of Na^+,K^+-ATPase have been identified on the basis of their ouabain sensitivity (Doucet & Barlet, 1986) and of their immunoreactivity with monoclonal antibodies (Barlet-Bas *et al.* 1993). Two isoforms of Na^+,K^+-ATPase with similar pharmacological and immunological properties have also been recognised in rat (Féraille *et al.* 1995*a*). However, contrasting with the segment-specific distribution of these isoforms of Na^+,K^+-ATPase observed in rabbit, the rat isoforms are co-expressed in comparable proportion all along the nephron. Thus, in the latter species, the mode of activation of the enzyme found in the collecting duct cannot be explained by a specific expression of isoenzymes. Segment-specific post-translational modifications of Na^+,K^+-ATPase offer an alternative explanation. Such a hypothesis is made likely by the fact that, in rat, as well as in rabbit (Barlet-Bas, Cheval, Khadouri, Marsy & Doucet, 1990*a*), the Na^+ sensitivity of Na^+,K^+-ATPase is twice as high in the collecting duct ($K_{0.5}$ for Na^+, 23 mM; Fig. 7) than in the proximal tubule ($K_{0.5}$ for Na^+, 46 mM; Féraille *et al.* 1994). This observation suggests that, in the collecting duct, the Na^+ affinity of Na^+,K^+-ATPase is already maximal under basal conditions and could not be further increased in response to hormones. The high Na^+ affinity of the enzyme in the collecting duct has two consequences: (1) a great sensitivity to autoregulation by intracellular Na^+, and (2) a capacity to respond to hormones by rapidly reabsorbing large amounts of Na^+ when V_{max} is increased, despite the relatively low abundance of the enzyme in this segment (Doucet *et al.* 1979).

The insulin-induced increase in the turnover rate of Na^+,K^+-ATPase suggests that, in the collecting duct cells, insulin produces a reversible modification of some intrinsic property of Na^+,K^+-ATPase, since this effect is lost after a permeabilization of the cells. Such observations have been previously reported in adipocytes (Resh, 1982; McGill, 1991) in which the insulin activation of the Na^+,K^+-ATPase was demonstrated in intact cells but not in plasma membrane or ghosts from insulin-pretreated adipocytes. These observations suggest that insulin induces or activates a cytosolic soluble factor which modulates Na^+,K^+-ATPase activity. An alternative explanation for the discrepancy between the increase in cation transport activity of Na^+,K^+-ATPase and the absence of alteration in its ATP hydrolytic activity is that insulin increases the efficiency of coupling between ATP hydrolysis and cation transport by the Na^+,K^+-ATPase. This reversibility of the insulin stimulation of Na^+,K^+-ATPase after permeabilization in collecting duct contrasts with its stability, under the same procedure, in PCT (Féraille *et al.* 1994). This finding further demonstrates the cellular specificity of the regulation of Na^+,K^+-ATPase.

In collecting duct as in proximal tubule, the insulin stimulation of Na^+,K^+-ATPase is a primary phenomenon independent of the rate of Na^+ entry into the cells. Blockade of amiloride-sensitive Na^+ channels and of Na^+–Cl^- cotransport (Fig. 1), as well as intracellular Na^+ depletion (Fig. 3) or Na^+ loading at saturation (Fig. 2) did not alter the stimulatory effect of insulin on the transport activity of Na^+,K^+-ATPase. This insulin-induced increase in Na^+,K^+-ATPase-mediated cation transport (Fig. 1) was not associated with changes in the intracellular Na and K concentrations when tubules were incubated with normal external Na^+ and K^+ concentrations (Fig. 5). This result implies that insulin must equally stimulate Na^+ entry and exit. This interpretation is supported by the observation that, in the proximal tubule, insulin stimulates both the apical Na^+–H^+ exchanger (Gesek & Schoolwerth, 1991) and Na^+,K^+-ATPase (Féraille *et al.* 1994). It is also in agreement with results obtained in A6 cells, a cell line derived from *Xenopus laevis* distal nephron which shares several properties of the principal cells of mammalian collecting duct. In these cultured cells, insulin activates and increases the density of the apical amiloride-sensitive Na^+ channels (Marunaka, Hagiwara & Tohda, 1992; Erlij, De Smet & Van Driessche, 1994). Altogether these observations strongly suggest that insulin controls both the apical and the basolateral steps of transepithelial Na^+ transport in epithelial kidney cells. This coordinated control of Na^+

transport systems would allow both a sustained increase in the transepithelial transport of Na^+, and maintain the intracellular Na^+ homeostasis in response to the hormone.

In summary, insulin primarily stimulates Na^+,K^+-ATPase in isolated rat collecting duct, as well as in rat proximal tubule. However, the mechanism of stimulation of the Na^+,K^+-ATPase possesses a cellular specificity. In the collecting duct, insulin enhances the maximal turnover rate of the Na^+,K^+-ATPase without changing the Na^+ affinity of the enzyme, whereas in PCT, the hormone increases the Na^+ affinity of the pump without modifying its V_{max}. This specificity is not explained by the presence of different isoforms of the Na^+,K^+-ATPase in these two segments. It might therefore be related to cell-specific regulatory mechanisms of the pump, as supported by the differences between proximal tubule and collecting duct in terms of: (i) Na^+ affinity of the Na^+,K^+-ATPase, and (ii) stability of the stimulatory process under permeabilization.

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Acknowledgements

We thank Dr A. Doucet for helpful discussion and criticism of the manuscript. This work was supported in part by the Swiss National Science Foundation (grant no. 3100-040-386.94-1).

Received 21 November 1994; accepted 17 March 1995.