The influence of intracellular sodium concentration ([Na+]i) on the number of Na+-K+-ATPase pumps was examined in cortical collecting tubules (CCD) of kidneys from rabbits in different aldosterone conditions. Specific [3H]ouabain binding was measured in isolated CCD with various [Na+]i. Experiments were performed on adrenalectomized rabbits receiving only a substitutional dose of dexamethasone and on adrenalectomized rabbits replete with aldosterone. In aldosterone-replete rabbits, the number of binding sites increased linearly with [Na+]i, from 16 fmol/nl tubular volume at 15 mM Na+, to 39 fmol/nl tubular volume at 140 mM Na+. Neither actinomycin D (5 μM) nor cycloheximide (10 μM) prevented this [Na+]i-dependent increase. In adrenalectomized rabbits, the number of ouabain-binding sites was reduced and did not increase with [Na+]i. These results are in favor of the presence of a "latent" pool of pumps in CCD, rapidly recruited under [Na+]i influence. Aldosterone appears to be required for the constitution and/or activation of this pool.

In vitro cell preparations, variations of the sodium load are currently realized by changing the intracellular sodium concentration (10, 19).

In this study, we established the relationship between intracellular sodium concentration and the number of basolateral pumps, as measured by specific [3H]ouabain binding, in isolated rabbit CCD. The influence of aldosterone on this relationship was studied using animals in various aldosterone conditions. Results show that, in the presence of aldosterone, [Na+]i induces a rapid increase in the number of Na+-K+-ATPase pumps that could be ascribed to the recruitment of pumps from a "latent" pool. In the absence of aldosterone, no sodium-dependent increase is observed, suggesting that this hormone is required for the constitution and/or activation of this pool.

Materials and Methods

Experiments were performed on female New Zealand rabbits. Kidneys were prepared for microdissection of isolated CCD. Five ml of blood was collected from aorta to measure plasma aldosterone concentration (radioimmunoassay, Compagnie ORIS Industrie, Saclay, France).

Microdissection of Isolated CCD—Microdissection of CCD (see Miniprint) was performed as previously described (20). After incubation of kidney pyramids with collagenase, CCD were dissected at 4°C in two different solutions depending upon the experiment: a saline solution was used for experiments on sodium-loaded tubules, and a sucrose solution was utilized in experiments on CCD in the absence of a sodium load. CCD were dissected in their "light" portion.

effect of aldosterone on this number (10, 19).
measured in CCD with various intracellular sodium concentrations. To increase [Na\(^+\)], CCD were microdissected in K\(^+-\)free saline solution (containing 139 mM NaCl, 10 mM NaHCO\(_3\), 0.8 mM MgSO\(_4\), 5 mM D-glucose, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 1 mM alanine, 20 mM HEPES/Tris, pH 7.4) and kept for various times (30 min to 4 h) at 4°C. As previously reported (21), this resulted in a gradual increase in [Na\(^+\)]. Then, tubules were transferred into the same K\(^+-\)-free saline solution containing 5 mM of \(^{22}\)Na (Amersham Corp.; 200 mCi/ml) and 2 x 10\(^{-5}\) M \[^{3}H\]ouabain. Tubules were incubated for 75 min at 20°C (see Miniprint).

After 75 min of incubation, CCD were rinsed in choline solution at 4°C for 15-30 min (see Miniprint), and double isotopic counting (\(^{22}\)Na and \(^{3}H\)) was performed.

Since both cell sodium and \[^{3}H\]ouabain binding were determined in single CCD by double isotopic counting, simultaneous measurements of extracellular sodium contamination ([Na\(^+\)]) and nonspecific \[^{3}H\]ouabain binding (NSB) were not possible on the same samples. Thus, for each experiment, [Na\(^+\)], and NSB were measured on separate samples. [Na\(^+\)], was determined by incubation of ~10-mm CCD in saline solution in the presence of \[^{22}\]Na and the extracellular marker \[^{3}H\]sorbitol (Du Pont-New England Nuclear; 24 Ci/nmol, 35 μCi). NSB was measured by incubation of ~10-15-mm CCD in saline solution in the presence of 2 x 10\(^{-5}\) M \[^{3}H\]ouabain and 2 x 10\(^{-7}\) M unlabeled ouabain. The mean value of [Na\(^+\)] in all experiments was 21 ± 3.7 peq/nl tubular volume and that of NSB was 1.1 ± 0.2 fmol/ nl tubular volume. For each individual CCD in which the total sodium content and total \[^{3}H\]ouabain binding were simultaneously measured, the values of NSB and [Na\(^+\)], determined in the same experiment were subtracted to obtain [Na\(^+\)], and specific \[^{3}H\]ouabain binding.

The effects of actinomycin D (5 μM) and cycloheximide (10 μM) were tested in three adrenalectomized rabbits receiving both dexamethasone and aldosterone; in two rabbits, either actinomycin D or cycloheximide was added to the incubation medium, and in one supplementary rabbit, both inhibitors were tested on separate tubular samples from the same kidney.

**Expression of Results**—In each experiment, CCD were photographed after rinsing in choline solution at 4°C using a camera placed on a stereomicroscope to determine the tubular volume of each sample (see Miniprint). Ouabain binding was expressed as femtomoles/nanoliter tubular volume. [Na\(^+\)], was expressed as picoequivalents/nanoliter tubular volume, i.e. as millimolar. The rational for expressing our results as a function of tubular volume are the following. First, it has been shown that the morphologic condition modulates cell volume (22); second, it is well-known that the steroid condition modulates cell sodium (21, 22); third, we observed that, even within the same kidney, large variations in tubular volume of CCD were present. Fig. 1 is an illustration of the influence of tubular volume on specific ouabain binding per unit tubular length: ouabain binding per millimeter tubular length under the same experimental condition is highly dependent on tubular volume.

**Results**

Plasma aldosterone concentration was 564 ± 123 pg/ml (n = 10) in sham-operated rabbits. For adrenalectomized rabbits (n = 9), all values of plasma aldosterone concentration were below the limit of sensitivity of the method (~50 pg/ml). The mean value was 675 ± 292 pg/ml in adrenalectomized rabbits receiving aldosterone (n = 5).

**Concentration Dependence of Ouabain Binding**—Fig. 2 shows the concentration dependence of total and nonspecific binding of \[^{3}H\]ouabain in sham-operated and adrenalectomized rabbits. In sham-operated rabbits, total binding saturates at ~10 fmol/nl tubular volume for 10\(^{-5}\) M ouabain. The apparent \(K_{d}\) was ~2 x 10\(^{-6}\) M. Nonspecific binding was very low and did not saturate up to 5 x 10\(^{-6}\) M ouabain. Similar patterns were observed in adrenalectomized rabbits: the apparent \(K_{d}\) was ~3.3 x 10\(^{-6}\) M. However, maximum total ouabain binding (~7.0 fmol/nl tubular volume) was lower than that in sham-operated rabbits. Nonspecific binding did not differ from that obtained in sham-operated rabbits. These results indicate that the number of specific binding sites is decreased by adrenalectomy, whereas the apparent affinity of Na\(^+-K\)-ATPase pumps for ouabain is not modified.

**Effect of [Na\(^+\)], on Specific Ouabain Binding in Presence of Na\(^+-K\)-ATPase Pumps**

![Fig. 1. Dependence of specific ouabain binding on tubular volume in CCD. Specific \[^{3}H\]ouabain binding is expressed per millimeter tubular length and plotted against the tubular volume per unit length. Each point is the mean value ± S.E. of 9-44 CCD. CCD were obtained from six kidneys of adrenalectomized rabbits, a condition in which sodium concentration does not affect specific ouabain binding (see "Results" and Fig. 5). \[^{3}H\]Ouabain concentration was 2 x 10\(^{-5}\) M. Very large variations in tubular volume are present within CCD. Specific ouabain binding is highly dependent on tubular volume (r = 0.92, n = 9, r = 0.95, p < 0.001; data obtained from 220 CCD).](image)

![Fig. 2. Concentration dependence of \[^{3}H\]ouabain binding. Upper, control sham-operated rabbits; lower, adrenalectomized (ADX) rabbits. Each point is the mean value ± S.E. of 4-38 individual CCD from three sham-operated and three adrenalectomized rabbits. CCD were incubated at 20°C in sucrose solution for 60 min with \[^{3}H\] ouabain in the presence or absence of a 100-fold excess of unlabeled ouabain. Nonspecific binding (NS) was low and similar under both conditions. The \(N_{max}\) was ~10 fmol/nl tubular volume in sham-operated rabbits, and 7 fmol/nl tubular volume in adrenalectomized rabbits. \(K_{d(app)}\) values were similar under both conditions (2.0 x 10\(^{-5}\) and 3.3 x 10\(^{-6}\) M).](image)
Aladosterone—The evolution of ouabain binding as a function of [Na⁺], for all experiments is illustrated in Fig. 3. Specific ouabain binding increased linearly with [Na⁺], from 16 fmol/ml tubular volume at 15 mM Na⁺, to 39 fmol/ml tubular volume at 140 mM Na⁺. The effects of the inhibitor of RNA synthesis, actinomycin D (5 μM), and of the inhibitor of protein synthesis, cycloheximide (10 μM), are illustrated in Figs. 4 and 6, respectively. It appears clearly that neither of these two inhibitors prevented the increase of specific ouabain binding with [Na⁺].

Effect of Adrenalectomy on [Na⁺]-dependent Specific Ouabain Binding—Fig. 6 illustrates the results obtained in adrenalectomized rabbits without aldosterone replacement. By contrast with data in the presence of aldosterone, no significant increase in specific ouabain binding occurred with increasing [Na⁺]. In addition, specific ouabain binding at low [Na⁺], was clearly lower than that obtained in the presence of aldosterone; at 15 mM Na⁺, specific ouabain binding was 7 fmol/nl tubular volume, as compared to 16 fmol/nl tubular volume in aldosterone-replete animals.

**DISCUSSION**

In CCD, an aldosterone-sensitive transporting epithelium which precisely adjusts the final reabsorption of sodium in the kidney (11), aldosterone enhances transepithelial sodium transport by increasing both sodium entry via apical channels and basolateral sodium extrusion via Na⁺-K⁺-ATPase pumps (12, 25). Since one of the initial effects of aldosterone consists of an increase in sodium entry into cells (12, 26) and since very large and rapid changes in Na⁺-K⁺-ATPase activity occur in this epithelium (31), we examined the possibility that the recruitment of latent pumps at the basolateral membrane could be dependent on cell sodium. For this purpose, both [Na⁺], and specific [H]ouabain binding were measured in isolated CCD from adrenalectomized rabbits with or without aldosterone infusion.

In the absence of external sodium (incubations in sucrose solution), concentration dependence curves of [H]ouabain binding show that the N₉₀ of Na⁺-K⁺-ATPase is reduced in adrenalectomized rabbits as compared to controls. This result is in accordance with previous reports (13). However, the affinity of Na⁺-K⁺-ATPase pumps for ouabain in control animals (Kₐ(app) = 2 × 10⁻⁹ M) is at variance with that
reported by Doucet and Barlet (1.2 × 10⁻⁷ m) in rabbit CCD (27). Differences in experimental conditions, such as a higher incubation temperature (37°C) and the use of vanadate, in the study of Doucet and Barlet may be the source of the difference in the observed affinity of Na⁺-K⁺-ATPase pumps for ouabain.

Whereas adrenalectomy reduced the N_max of Na⁺-K⁺-ATPase pumps, the affinity of basolateral pumps for ouabain was unchanged by adrenalectomy, as evidenced by the similar K_d(app) values obtained in CCD from sham-operated and adrenalectomized rabbits.

The simultaneous determination of specific [³H]ouabain binding and [Na⁺], in individual CCD clearly demonstrates that the number of basolateral pumps closely depends on [Na⁺], in animals receiving aldosterone. Cell sodium loading is a method currently used to examine the effects of sodium on Na⁺-K⁺-ATPase in in vitro cell preparations (10, 19). Such studies are based on the assumption that [Na⁺], reflects the sodium load that interacts with the pump. In transporting epithelia, such as CCD, the sodium load may vary within a large range, depending on the sodium delivery at the luminal membrane, as reflected by changes in transepithelial sodium transport (18). In our experiments, the number of pumps increased two to three times between 15 and 140 mM Na⁺, (Fig. 3). This increase was not dependent on RNA or protein synthesis since neither actinomycin D nor cycloheximide suppresses it (Figs. 4 and 5). The relatively short delay for the [Na⁺],-dependent increase in the number of pumps, associated with the absence of an effect of inhibitors of both RNA and protein synthesis, strongly supports the notion of a recruitment of pre-existent latent pumps under the influence of [Na⁺],. Such a notion was recently evoked in view of the increase of ouabain binding after incubation of rat CCD with [Na⁺],. Such a notion was recently evoked in view of the observed affinity of Na⁺-K⁺-ATPase pumps, the affinity of basolateral pumps for ouabain was unchanged by adrenalectomy, as evidenced by the similar K_d(app) values obtained in CCD from sham-operated and adrenalectomized rabbits.

In adrenalectomized animals without aldosterone repletion, specific ouabain binding at 15 mM Na⁺, was lower than in aldosterone-replete animals. This is in accordance with data obtained after incubation in sucrose solution (Fig. 2). In addition, an increase in [Na⁺], up to 140 mM did not result in a recruitment of basolateral pumps, suggesting that the effect of [Na⁺], requires the presence of aldosterone.

In conclusion, this study indicates that, in rabbit CCD, a pool of latent pumps is present in the cell. These pumps are rapidly mobilized at the basolateral membrane under the influence of intracellular sodium. Whether the latent pool is composed of inactive pumps in the membrane or of intracellular pumps warrants further studies. In aldosterone-depleted rabbits, the N_max of specific ouabain binding is reduced, and no [Na⁺],-dependent recruitment of pumps is observed. This suggests that aldosterone is necessary for the constitution of this latent pool and/or its activation.

REFERENCES


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Concentration-dependency of ouabain binding was established in adrenalectomized and control sham-operated rabbits. Bilateral adrenalectomy was performed through an abdominal incision, under anesthesia (80% nitrogen peroxide - 20% oxygen, in flurane). In order to improve the condition of rabbits just after surgery, they received desyrdrococtaminate (Synoryt, Roussel Uclaf, France) 1.9 mg per kg b.w. the day of the experiment. sham animals were fed a standard laboratory diet. Sham had free access to tap water until experiments and Ad animals 0.9% NaCl solution. Experiments were performed five days after surgery.

In order to examine the relationship between intracellular Na concentration and [3H]-ouabain binding, and the influence of aldosterone on this relationship, experiments were done on adrenalectomized rabbits, in the presence or absence of aldosterone. These Ad rabbits did not receive synoryt. They were continuously infused with low doses of desyrdrococtaminate from the time of adrenalectomy up to the experiment. This was realized using osmotic minipumps (Alzet 2001), Alzet Corp - Palo Alto, CA) placed subcutaneously and delivering 1 μg per 100 g per day of desyrdrococtaminate. This dose has been demonstrated to yield pharmacological concentration of glucocorticoid, without mineralocorticoid-like effects. Six animals received only desyrdrococtaminate. To test the effect of aldosterone, five minipumps were added, in addition to desyrdrococtaminate, 3 μg per 100 g per day aldosterone (via minipumps). Experiments were performed five days after surgery.

Using ouabain, each point is the mean value ± SE of 4 to 12 CCD. Maximal binding was reached after 10 min with 2.7 x 10^-5 M [3H]-ouabain. After preincubation of 20°C with high NaCl (40 mM) and 0.05% collagenase, or without 2 x 10^-3 M unlabeled ouabain, after preincubation at 4°C in K-free saline or sucrose solution. Each point is the mean value ± SE of 4 to 12 CCD. After incubation, CCD were rinsed for various times in choline solution at 4°C. Total binding did not change with the duration of rinsing between 15 to 90 min. Total binding was higher in presence than in absence of Na in solutions. Non specific binding represents a small percentage of total binding so early as after 15 min rinsing time.

Cell sodium loading, the inclusion time necessary to obtain isometric equilibration between NaCl of Na-loaded tubules and the external solution containing 22Na was determined for this purpose, kinetics of isotonic equilibration were performed. CCD were Na-loaded at 4°C in saline solution and then incubated for different times at 20°C in the presence of [22Na] and [3H]-outubain. Isometric equilibration was reached in 45 mm for CCD with moderate Na, NaCl of Na-loaded tubules and the external solution containing 22Na was determined for this purpose, kinetics of isotonic equilibration were performed. CCD were Na-loaded at 4°C in saline solution and then incubated for different times at 20°C in the presence of [22Na] and the specific [3H]-ouabain binding was determined in the presence of 2.7 x 10^-5 M [3H]-ouabain with or without 2 x 10^-3 M unlabeled ouabain, after preincubation at 4°C in K-free saline or sucrose solution. Each point is the mean value ± SE of 4 to 12 CCD. After incubation, CCD were rinsed for various times in choline solution at 4°C. Total binding did not change with the duration of rinsing between 15 to 90 min. Total binding was higher in presence than in absence of Na in solutions. Non specific binding represents a small percentage of total binding so early as after 15 min rinsing time.
Recruitment of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase Pumps

**FIG. 5. Absence of effect of intracellular Na\textsuperscript{+} concentration on extracellular Na\textsuperscript{+} concentration and non-specific ouabain binding.** CCD from 4 kidneys of sham-operated rabbits were presoaked for various times in K\textsuperscript{-}free saline solution at 4°C. Thereafter, CCD were transferred either in saline solution with 22Na and \textsuperscript{3}H-ouabain (upper panel) or in saline solution with \textsuperscript{2}HNa and \textsuperscript{3}H-ouabain (2 x 10\textsuperscript{-9} M) plus unlabelled ouabain (2 x 10\textsuperscript{-3} M) (lower panel). Extracellular Na\textsuperscript{+} concentration (Na\textsubscript{e}) or non-specific ouabain binding were plotted against Na\textsubscript{e}. No effect of Na\textsubscript{e} was visible on Na\textsubscript{e} (y = 0.05x + 22.9), a = 0.01, r < 0.75, NS) or non-specific ouabain binding (y = 7.61 x 10\textsuperscript{-4} x + 1.47, n = 61, r = 0.01, NS).

**Determination of tubular volume:** the tubular volume of each individual CCD was measured as follows: CCD were photographed using a camera placed on a stereomicroscope. Tubular surface area and tubular length were determined using an image analyzer (Biocom, France). Since it has been shown that tubular lumen of non-perfused isolated CCD represents less than 5% of tubular volume\textsuperscript{5}, tubular volume was calculated assuming a cylindrical shape.

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