Enhanced Intracellular Sodium Concentration in Kidney Cells Recruits a Latent Pool of Na-K-ATPase Whose Size Is Modulated by Corticosteroids*

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Besides its role in the control of the rate of functioning of each Na-K-ATPase unit (as a substrate of the enzyme), the intracellular sodium concentration also regulates the number of active Na-K-ATPase units, as previously described in cultured cells. To evaluate such a possibility in kidney epithelial cells, the intracellular concentration of sodium in rat cortical collecting tubules (CCT) maintained in vitro was altered by the use of the sodium ionophore nystatin. When CCT were preincubated for 2-3 h at 37 °C in the presence of nystatin, the enzymatic activity of Na-K-ATPase was markedly stimulated as compared to tubules preincubated without nystatin or in the presence of the ionophore but in the absence of extracellular sodium. Although nystatin increased both Na-K-ATPase activity and [3H]ouabain specific binding in CCT, its action was independent of de novo synthesis of the pump since neither actinomycin D nor cycloheximide abolished it. It is suggested that increasing the sodium concentration in CCT cells induces the recruitment of a latent pool of Na-K-ATPase units. The size of this latent pool of enzyme is under the control of corticosteroids as it is markedly decreased in CCT from adrenalectomized rats.

In the collecting tubule of the mammalian nephron, the capacity for sodium reabsorption varies within a wide range in response to several hormones and/or factors (1). In these cells, sodium reabsorption from the luminal medium to the peritubular fluid is accomplished through a passive luminal entry via amiloride-sensitive sodium channels (2) and active basolateral pumping catalyzed by Na-K-ATPase (Na+/K+-transporting ATPase, EC 3.6.1.37). Under normal conditions, the activity of Na-K-ATPase balances the sodium influx through the luminal membrane to maintain the constancy of the intracellular sodium concentration. When luminal sodium conductance increases, the intracellular sodium concentration rises and stimulates, by a substrate effect, the activity of each catalytic unit of Na-K-ATPase. If this increase of sodium entry is sustained, the sodium efflux through the pump can balance the sodium influx through the luminal membrane only if Na-K-ATPase remains stimulated, i.e. if the intracellular sodium concentration remains elevated. To maintain a normal intracellular sodium concentration with an increased pumping rate, one possibility is to enhance the V\textsubscript{max} of Na-K-ATPase.

The purpose of our study was to investigate the mechanisms through which the V\textsubscript{max} of the enzyme may increase and, in particular, whether an increase in the intracellular sodium concentration may stimulate Na-K-ATPase. For this purpose, changes in the intracellular sodium concentration were induced in microdissected rat cortical collecting tubules (CCT) by the use of the polyanion antibiotic nystatin, a sodium ionophore (3). Since stimulation of the V\textsubscript{max} of an enzyme may result from increasing either the number of active catalytic units or the activity of each catalytic unit, or both, we determined the number of active pump units present in collecting tubules incubated in the absence or presence of nystatin by measuring the specific binding capacity of [3H]ouabain. The use of an in vitro system allowed us to further evaluate the molecular events underlying the action of an increased intracellular concentration on Na-K-ATPase.

Finally, the possible relationship between adrenal steroids, which are known to induce Na-K-ATPase synthesis in rat collecting tubule (4, 5), and increased intracellular sodium concentration was investigated.

**EXPERIMENTAL PROCEDURES**

**Animals**—Experiments were carried out on normal or adrenalectomized male Wistar rats weighing 150-200 g and fed the usual laboratory diet ad libitum. Adrenalectomy was performed under light ether anesthesia 12 h to 7 days before study. Adrenalectomized rats had free access to a 0.9% NaCl solution for drinking, whereas normal rats were given tap water.

**Isolation of Single Pieces of Cortical Collecting Tubules**—After anesthesia (pentobarbital, 5 mg/100 g of body weight), the left kidney was quickly perfused via the abdominal aorta with 4 ml of incubation solution (see composition below) containing 0.3% (w/v) collagenase. To reduce kidney anoxia, the aorta was ligated above the left renal artery juncion only just before starting the perfusion. The left kidney was immediately removed and sliced into small pyramids which were incubated at 30 °C for 15-20 min in aerated incubation solution containing 0.07% (w/v) collagenase and then thoroughly rinsed in ice-cold incubation solution and kept at 4 °C.

**Cortical portions of collecting tubule were microdissected in incubation solution maintained at 4 °C. Tubules were then individually transferred with 0.5 ml of incubation solution to the cavities of a sunken bacteriological slide coated with dried bovine serum albumin. The length of each tubule (0.3-1.2 mm) was measured by automatic image processing as previously reported (6).

The incubation solution, derived from Eagle's minimal essential medium, contained 120 mM NaCl, 5 mM KCl, 1 mM CaCl\textsubscript{2}, 1 mM MgSO\textsubscript{4}, 4 mM NaH\textsubscript{2}PO\textsubscript{4}, 4 mM NaHCO\textsubscript{3}, 5 mM glucose, 10 mM lactate, 1 mM pyruvate, ~4 mM essential and nonessential amino acids, ~3.10\textsuperscript{3} mM vitamins, and 20 mM HEPES. Dextan 40,000 (0.3%, w/v) and bovine serum albumin (0.1%, w/v) were added. The

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1 The abbreviations used are: CCT, cortical collecting tubule(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
pH of the final solution was adjusted to 7.5.

Alteration of Intracellular Sodium Concentration—Increasing the intracellular sodium concentration was achieved by incubating individual segments of CCT in the presence of the sodium ionophore nystatin. At the concentration of 0.1 unit/μl used in this study, nystatin increased the intracellular sodium concentration from 25 to 140 mM within 5-10 min at 37 °C.

Thus, after addition to each tubule sample of another 0.5 μl of incubation solution which contained nystatin (0.2 unit/μl) or its solvent, incubation at 37 °C was started and lasted for 15 min to 3 h. When necessary, pharmacological agents were added to this solution at a concentration twice that required in the final medium.

After incubation, Na-K-ATPase activity and [3H]ouabain specific binding were measured according to the previously described microtechniques (7, 8) as summarized below.

Measurement of Na-K-ATPase Activity—ATPase activity was determined under Vmax conditions by a radiochemical microassay (measurement of "P2 released from [γ-32P]ATP) after permeabilization of cell membranes (7). Na-K-ATPase activity was taken as the difference between the mean activities of total ATPase (determined in the presence of sodium and potassium) and of Mg-ATPase (determined in the absence of sodium and potassium and in the presence of ouabain), each being measured on four to five replicates.

Measurement of [3H]Ouabain Specific Binding—In contrast to Na-K-ATPase activity, [3H]ouabain binding was measured on intact tubules by the low dead-space technique (9). Due to the low sensitivity of rat kidney to cardiac glycosides, saturation of the specific sites of binding was obtained only with very high concentrations of [3H]ouabain above 200 μM (9). Using such high concentrations of ouabain led to a rather important nonspecific binding (determined in the presence of 5 mM unlabeled ouabain and 90 mM KCl) which accounted for 60-75% of the total binding. Despite this difficulty, the number of [3H]ouabain specific binding sites was reproducibly measured at saturation.

Measurement of [35S]Methionine Incorporation—[35S]Methionine incorporation into proteins was determined in single segments of CCT to assess the efficiency of cycloheximide treatment. For this purpose, segments of CCT were individually preincubated at 37 °C for 1 h in 1 μl of incubation solution in the presence or absence of 20 μM cycloheximide. After addition of another 1 μl of incubation solution containing [35S]methionine (Amersham Corp.; >1300 Ci/mmol, 1-2 × 106 cpm/sample) in the presence or absence of cycloheximide and/or nystatin, tubules were incubated for an additional 2 h at 37 °C before addition of 10 μl of trichloroacetic acid (10%, w/v) and washing. Washing consisted of aspirating the piece of tubule with 0.2 μl of incubation solution into a polyethylene tube and transferring it into 100 μl of incubation solution. After brief stirring, this maneuver was repeated twice, and the piece of tubule was finally transferred with 0.2 μl of the final washing solution into a counting vial containing 0.5 ml of 1% (w/v) deoxycholate. The radioactivity was determined after adding 5 ml of scintillation fluid. In addition, the radioactivity of washing solutions, which consisted of 0.2 μl of the final washing solution, was used in each assay to determine the extracellular contamination with [35S]methionine. Under these conditions, incorporation of [35S]methionine into trichloroacetic acid-precipitable material increased linearly with incubation time for at least 3 h (data not shown).

Statistics—Statistical comparison between the various groups was performed by Student's t test for unpaired data or, when necessary, by variance analysis according to the test of Dunnett (10), with p values less than 0.05 being considered significant.

RESULTS AND DISCUSSION

Stimulation of Na-K-ATPase Activity in CCT from Normal Rats by Increased Intracellular Sodium Concentration—Results depicted in Fig. 1 indicate that incubation of CCT from normal rats for 3 h in the presence of nystatin (0.1 unit/μl) induced a marked stimulation of Na-K-ATPase activity (control, 880 ± 48 pmol-mm⁻¹-h⁻¹; nystatin, 1443 ± 63 pmol-mm⁻¹-h⁻¹; mean ± S.E., n = 19, p < 0.001). When added during Na-K-ATPase assay, nystatin had no effect on enzyme activity (data not shown). Na-K-ATPase activity was also stimulated after preincubation of CCT in the presence of amphotericin B (1 μg/ml), another sodium ionophore (Fig. 2). Similarly, preincubation of CCT in the presence of 1 mM ouabain, a procedure also aimed at increasing the intracellular sodium concentration, stimulated Na-K-ATPase activity (determined after washing off ouabain) (Fig. 2). Furthermore, the stimulatory action of nystatin was totally abolished when choline was substituted for sodium in the incubation solution (Fig. 3). These results demonstrate that stimulation of Na-K-ATPase maximal activity is not due to a direct effect of nystatin. Rather, it is likely related to alterations in the intracellular sodium (or potassium) concentration. Alternatively, an increase in Na-K-ATPase activity in response to nystatin could conceivably result from a rise in the intracellular calcium ion concentration through a secondary inhibition of Na⁺/Ca²⁺ exchange. However, in one experiment in which we attempted to increase the intracellular Ca²⁺ concentration by preincubating CCT for 3 h in the presence of the calcium ionophore ionomycin (1.7 × 10⁻⁸ M) and Ca²⁺ (1 mM), Na-K-ATPase activity did not increase, whereas it did in response to ouabain and nystatin in the same experiment (control, 795 ± 186 pmol-mm⁻¹-h⁻¹; ionomycin, 673 ± 155 pmol-mm⁻¹-h⁻¹; ouabain, 1536 ± 218 pmol-mm⁻¹-h⁻¹; nystatin, 1370 ± 308 pmol-mm⁻¹-h⁻¹; mean ± S.D.).
The mechanism of stimulation may prevail in CCT cells. We first determined whether stimulation of Na-K-ATPase activity reflected alterations in the number of catalytic units or the activity of individual units, or both. Results in Fig. 6A clearly indicate that preincubation of CCT in the presence of nystatin increased both Na-K-ATPase activity and specific binding of [3H]ouabain and by a similar factor. Consequently, the specific activity of Na-K-ATPase, i.e. the turnover rate of each pump unit under V_{max}, conditions, was not altered by nystatin and was similar to that previously reported for rabbit CCT (8). That nystatin altered the binding capacity and not the affinity of collecting tubules for ouabain was assessed by the finding that the apparent K_i of Na-K-ATPase for ouabain was similar with and without nystatin pretreatment (Fig. 5B). At this point, it should be stressed that in these experiments, ouabain binding was measured in intact nonpermeabilized cells so that only ouabain-binding sites accessible in the plasma membrane were detected. Thus, stimulation of Na-K-ATPase in response to nystatin involves the setting in place of new catalytic sites.

In vitro up-regulation of Na-K-ATPase was reported in several cell types (reviewed in Ref. 17) in response to an increased intracellular sodium concentration brought about by growing cells in low potassium medium or in the presence of either sublethal doses of ouabain or veratridine (in those cells which have voltage-sensitive sodium channels). Such stimulation of Na-K-ATPase peaked after about 24 h. It corresponded to an induction of Na-K-ATPase synthesis, as assessed by increased incorporation of [35S]methionine into immunoprecipitable α-subunits of Na-K-ATPase in myocytes (16), enhanced rates of synthesis of α- and β-mRNAs in Madin-Darby canine kidney cells (12), and/or an inhibition of the degradation rate of pre-existing pump units (15, 16).

We therefore evaluated whether stimulation of Na-K-ATPase activity in response to nystatin was brought about by increased synthesis of Na-K-ATPase. Results in Fig. 6 indicate that neither actinomycin D nor cycloheximide affected the effect of nystatin when added concomitantly to it. Although these drugs were shown to efficiently inhibit Na-K-ATPase synthesis in rat CCT when used under similar conditions (4), one might argue that their action could be altered in the presence of nystatin. To exclude this possibility, we evaluated the effect of cycloheximide on [35S]methionine incorporation in single CCT incubated in the presence or absence of nystatin. Data in Fig. 7 indicate that under these two conditions, cycloheximide inhibited by 95% [35S]methionine incorporation into proteins of CCT. They also indicate that nystatin alone had no detectable effect on [35S]methionine incorporation. In parallel experiments, we verified that cycloheximide did not alter nystatin-induced stimulation of Na-K-ATPase (Fig. 7). Thus, up-regulation of Na-K-ATPase observed in rat CCT in response to nystatin cannot be accounted for by increased synthesis of Na-K-ATPase, neither can it be attributed to a decreased rate of Na-K-ATPase degradation, as its fast kinetics (see Fig. 4) is incompatible with the half-life (~15 h) of the enzyme (18). Results from these experiments instead suggest that an increased intracellular sodium concentration triggers the rapid redistribution of pre-existing inactive enzyme molecules to the plasma membrane of CCT cells. Presently, we have no information concerning the intracellular localization and the molecular form of this latent pool of Na-K-ATPase. This hypothesis does not preclude that other mechanisms, including stimulation of Na-K-ATPase synthesis, may occur later in CCT cells, especially since these cells share several properties with the Madin-Darby canine kidney cell line, in which stimulation of the transcription of genes encoding for Na-K-ATPase subunits was reported in response to an enhanced intracellular sodium level (12).

**Control of Size of Latent Pool of Na-K-ATPase by Cortico-**
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A 6 Na-K-ATPase activity

0 25 50 75 100

Specific 3H ouabain binding

0 5 10

Na-K-ATPase specific activity

0 1000 2000

FIG. 5. Effect of nystatin on Na-K-ATPase activity, specific binding of [3H]ouabain, and sensitivity to ouabain. A, cortical collecting tubules from normal rats were preincubated for 2.5 h at 37°C in the presence (hatched bars) or absence (open bars) of 0.1 unit/μl nystatin before measurement of both Na-K-ATPase activity (nanomoles·millimeter⁻¹·hour⁻¹) and (3H)ouabain specific binding (femtomoles·millimeter⁻¹). Na-K-ATPase specific activity (minute⁻¹) was calculated as the ratio of the activity over the number of ouabain-binding sites. Values are means ± S.E. of four separate experiments. *, p < 0.05 as compared to control by Student's t test. B, ATPase activity was measured in the presence or absence of various concentrations of ouabain in cortical collecting tubules from normal rats after a 2.5-h preincubation at 37°C in the presence (O) or absence (●) of 0.1 unit/μl nystatin. Values are means ± S.E. of four to five samples from the same rat.

Fig. 6. Effects of inhibitor of protein synthesis on stimulation of Na-K-ATPase by nystatin. Tubules from the same animals were incubated for 3 h at 37°C in the presence (hatched and stippled bars) or absence (open bars) of 0.1 unit/μl nystatin either under normal conditions (control) or in the presence of 5 μM actinomycin D or 20 μM of cycloheximide. Values are means ± S.E. of three to four separate experiments. Differences statistically significant between groups were determined by variance analysis (*, p < 0.05; **, p < 0.005; NS, not statistically different).

Fig. 7. Effect of nystatin and cycloheximide on [35S]methionine incorporation into proteins. Cortical collecting tubules from normal rats were incubated for 3 h at 37°C in the presence or absence (control) of 20 μM cycloheximide. After the first hour, 0.1 unit/μl nystatin was added to half the samples (hatched and stippled bars), whereas the same volume of incubation solution was added to the other half (open bars). For measurement of [35S]methionine incorporation in trichloroacetic acid-precipitable material (left), [35S]methionine was added during the last 2 h of incubation (i.e. at the same time as nystatin or diluent). For Na-K-ATPase measurements (right), no [35S]methionine was added. Values are means ± S.E. from five animals.

steroids—In contrast to what was observed in normal rats, preincubation of CCT in the presence of nystatin did not alter Na-K-ATPase activity in tubules isolated from adrenalectomized rats (adrenalectomized, 199 ± 47 pmol·mm⁻¹·h⁻¹; adrenalectomized + nystatin, 213 ± 43 pmol·mm⁻¹·h⁻¹; mean ± S.E., n = 6, not significantly different), suggesting that there is no latent pool of Na-K-ATPase in CCT of adrenalectomized animals (Fig. 8, inset). This is consistent with the previous finding that preincubation with ouabain for 6 h did not increase Na-K-ATPase activity in suspensions of outer medullary collecting tubules from adrenalectomized rats (19). To study the kinetics of decay of the latent pool of pumps following adrenalectomy, Na-K-ATPase activity of CCT incubated for 2 h in the absence or presence of nystatin was determined at various times (from 12 h to 1 week) after adrenalectomy. Results in Fig. 8 indicate that Na-K-ATPase activity measured after incubation without nystatin (activity of the pool of active Na-K-ATPase) remained constant during the first 24 h following adrenalectomy and then decreased to the values observed 1 week after adrenalectomy. In contrast, Na-K-ATPase activity measured after stimulation by nystatin, which corresponds to both active and latent pools of Na-K-ATPase, decreased as soon as 12 h following adrenalectomy and equaled the unstimulated activity at 1 day after adrenalectomy and thereafter. These results suggest that the latent
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FIG. 8. Evolution of nystatin action after adrenalectomy. Cortical collecting tubules microdissected from normal rats (day 0) or from rats adrenalectomized for 0.5 to 6–7 days were incubated at 37°C for 2 h in the presence (•) or absence (○) of 0.1 unit/ml nystatin before Na-K-ATPase measurement. Each point is the mean ± S.E. of six to eight experiments. The difference between the two curves (- - -) shows the evolution of the size of the latent pool of Na-K-ATPase after adrenalectomy. Inset, CCT from normal or 6–7-day adrenalectomized (ADX) rats were preincubated for 2 h at 37°C in the presence (□) or absence (○) of nystatin before Na-K-ATPase measurement. Each line joins the experimental values determined in a given animal. Points are means ± S.E. for the two conditions. *, p < 0.001 as compared to control by Student’s t test.

pool of Na-K-ATPase, as estimated by the difference between nystatin-stimulated and -unstimulated activities, rapidly disappears during the first 24 h following adrenalectomy. Thus, the constancy of the unstimulated Na-K-ATPase activity during the first 24 h following adrenalectomy is due to a progressive recruitment of the latent pool of Na-K-ATPase. This recruitment could be induced by a transient increase in the intracellular sodium concentration brought about by the disappearance of active pump units in the basolateral membrane.

Aldosterone has been demonstrated to stimulate Na-K-ATPase activity in CCT from adrenalectomized animals by inducing de novo synthesis of active enzyme units (4, 5). Our results suggest that corticosteroids also participate in replenishing the latent pool of Na-K-ATPase. Thus, the question arises whether newly synthetized pump units must transit through the latent pool before being targeted into basolateral membrane of collecting tubule cells. If so, we have to admit that stimuli other than the intracellular sodium concentration may induce the recruitment of the pool since in CCT from adrenalectomized rats, aldosterone induces the appearance of new pump units independently of alterations in the intracellular sodium level (4).

Our hypothesis is that the latent pool of Na-K-ATPase serves as a reservoir of pump units which can be rapidly recruited and thereby may effectively compensate ionic imbalance created by increasing sodium entry in CCT cells. It will be of importance to determine: 1) whether such a pool exists in other nephron segments submitted to large changes in transport capacity, and 2) whether intracellular sodium is the only parameter controlling its recruitment.

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