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Effect of Ca^{2+} on the ouabain-insensitive, active Na^+ uptake in inside-out basolateral plasma membrane vesicles from rat kidney proximal tubular cells

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The ouabain-insensitive, active Na^+ uptake of inside-out vesicles prepared with basolateral plasma membranes from rat kidney proximal tubular cells can be increased by the presence of micromolar concentrations of Ca^{2+} in the assay medium. The concomitant ATP hydrolysis associated with the Na^+ uptake is also increased by the presence of Ca^{2+}. The Na^+ uptake and the concomitant ATP hydrolysis are inhibited by 2 mM furosemide. The effect of Ca^{2+} is not due to the activity of an Na^+-Ca^{2+} exchanger. The present results are in accordance with our previous model (Proverbio, F., Proverbio, T. and Marín, R. (1982) Biochim. Biophys. Acta 688, 757–763) in which we proposed that Ca^{2+} seems to modulate the activity of the ouabain-insensitive Na^+ pump, in two different ways: (1) in a strong association with the membranes in which Ca^{2+} (stable component) is essential for the pump activity and (2) in a weak association with the membranes in which Ca^{2+} (labile component) can be quickly and easily removed by reducing the free Ca^{2+} concentration of the assay medium to values lower than 1 μM. The K_a for Ca^{2+} (for the labile component) is around 5 μM. The Ca^{2+} modulation of the ouabain-insensitive Na^+ pump is an indication that Ca^{2+} could regulate the magnitude of the Na^+ extrusion accompanied by Cl^- and water present in rat kidney proximal tubular cells.

Introduction

An ouabain-insensitive Na^+-stimulated ATPase (Na^+-ATPase) has been demonstrated in basolateral plasma membranes from cells of mammalian renal cortex [1,2]. This Na^+-ATPase is totally inhibited by furosemide and triflocin and is insensitive to the presence of K^+ in the assay medium [1–3]. When kidney cortex slices are rewarmed in a medium with K^+, after leaching in the cold in a medium without K^+ (to load them with Na^+), Na^+ is extruded in exchange for K^+ and is extruded along with Cl^- and water. The first mode of Na^+ extrusion is K^+-dependent, ouabain-sensitive and furosemide- and triflocin-insensitive. The latter mode of Na^+ extrusion is K^+-independent, ouabain-insensitive and furosemide- and triflocin-sensitive [4–8]. The parallelism between the characteristics of the Na^+-ATPase and the Na^+ extrusion (accompanied by Cl^- and water) has been taken as an indication that the Na^+-ATPase is the biochemical manifestation of this mode of Na^+ extrusion [4–8].

During the last few years, several approaches were utilized to demonstrate the Na^+-ATPase: ageing of microsomal preparations at 4°C [9], resuspension of basolateral plasma membrane preparations at pH 7.8 [1] and determination of the ATPase activity in the presence of micromolar quantities of Ca^{2+} [1]. We studied in more detail the activating effect of Ca^{2+} on the Na^+-ATPase [10] and it was found that Ca^{2+} modulates the activity of the Na^+-ATPase depending on its as-

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sociation to the membranes: (1) Ca^{2+} strongly associated to the membranes is essential for the ATPase activity and (2) Ca^{2+} weakly associated to the membranes which increases the V_{max} of the ATPase.

In a recent paper [11], working with inside-out vesicles of basolateral plasma membranes prepared from rat kidney proximal tubular cells, we were able to demonstrate an ouabain insensitive Na^+ uptake that is inhibited by 2 mM furosemide. This Na^+ uptake is accompanied by ATP hydrolysis and appears to be against a concentration gradient. The characteristics of this Na^+ uptake clearly differentiate it from cotransport systems like the Na^+-Cl^- or the Na^+-K^+-$2Cl^-$. It was concluded that the Na^+-ATPase is able to support the active transport of Na^+ across the basolateral plasma membranes of the proximal tubular cells.

The present work was undertaken to study the modulatory effect of Ca^{2+} on the ouabain-insensitive active Na^+ uptake in inside-out basolateral plasma membrane vesicles prepared from rat kidney proximal tubular cells. The present results support our previous findings with the Na^+-ATPase that Ca^{2+} modulates the activity of the ouabain-insensitive Na^+ pump in two different ways.

Materials and Methods

Preparation of fractions enriched in inside-out basolateral plasma membrane vesicles

Outermost slices of kidney cortex (which are known to be rich in proximal tubules) of healthy male Sprague-Dawley rats of 3 months of age were obtained as already described [2,11]. The slices were homogenized at 4°C in 3 vol. of 150 mM sucrose/50 mM NaCl/2 mM ouabain/20 mM Tris-HCl (pH 7.2) with eight strokes at 2500 rpm using an Eberbach homogenizer with a Teflon pestle. The homogenate was centrifuged as previously described [11]. The treatment of the tissue included 2 mM ouabain in all the solutions. The final pellet was resuspended in the sucrose/Na^+/ouabain/Tris medium described to give a protein concentration of about 3–4 mg/ml. The purity of the fraction was tested by the determination of different enzymatic markers as described before [2,11]. Before the Na^+ uptake experiments, samples of the fractions were asssayed for the percentage of inside-out vesicles by determining their ($Na^+ + K^+$)-ATPase activities before and after treating the membranes with either 6.25 μg valinomycin/mg protein or 0.06% deoxycholate and 2 mM EDTA (for details see Refs. 11, 12).

Na^+ uptake by the vesicles

The method already described was followed [11]. Briefly, the vesicles were preincubated for 2 h at 0°C in the different media which contained (mM): NaCl, 50, with 50 μCi/ml ^{24}Na; Tris-HCl (pH 7.2), 10; sucrose, in sufficient quantity to obtain an osmolarity of 300 mosmol/l; ATP, 2; ouabain, 2 and according to the experimental design: furosemide, 2; ionophore A23187, 0.002. The quantity of Ca^{2+} added to each incubation medium to obtain the desired free Ca^{2+} concentration was determined in parallel experiments. This determination consisted of the addition of known amounts of Ca^{2+} to the specific incubation medium with Mg^{2+}, measuring simultaneously the free Ca^{2+} concentration with an Orion electrode. After preincubation, the suspensions were warmed to 37°C and incubations were started by the addition of 5 mM $MgCl_2$ and carried out for 1 min. Control tubes were run in the absence of Mg^{2+} with 0.5 mM EDTA. The final volume of the reaction was 120 μl with about 40 μg total protein. Each point represents triplicate determinations. The protein content of the original suspensions was measured by the method of Lowry et al. [13]. At the end of the experiment, 100 μl of the incubation mixture were passed through a $Tris^+$-equilibrated Dowex 50W-X8 resin column in order to separate the vesicles from the incubation medium. The column was immediately washed with 3 ml 250 mM sucrose/5 mM $MgCl_2$/20 mM Tris-HCl (pH 7.2). The eluates were collected in counting vials and the radioactivity was determined. The Na^+ uptake by the vesicles is expressed in nmol/mg protein per min after correction for Na^+ binding [11]. In previous experiments, we have found that 50 μM Ca^{2+} does not affect the Na^+ binding (data not shown). The preincubations of the vesicles with EGTA were carried out at 0°C for 3–4 h in the same sucrose/Na^+/ouabain/Tris medium to which 2 mM EGTA was added. Before the Na^+ uptake experiments, the vesicles treated

with EGTA were washed at 4°C with the sucrose/Na^+/ouabain/Tris medium in order to remove the EGTA.

ATP hydrolysis during Na^+ uptake

Vesicle suspensions (about 200 μg total protein), were preincubated in the same way as for the Na^+ uptake experiments but without ^{24}Na. After the preincubation period, the suspensions were warmed to 37°C and the incubations were started by the addition of 5 mM $MgCl_2$. The final volume of the reaction was 600 μl and at the end of the incubation periods 1 ml of ice-cold $HClO_4$ was added to the incubation tubes. The liberated phosphate was determined in the deproteinized solution according to the method of King [14]. The ATP hydrolyzed is expressed as nmol phosphorus liberated per mg protein in 1 min incubation. The ATP hydrolysis not associated with the active Na^+ uptake (Mg^{2+}-ATPase) was measured in parallel experiments without adding Na^+ to the incubation medium and in the presence of 2 mM furosemide. Furosemide is able to inhibit all the Na^+-ATPase without affecting the Mg^{2+}-ATPase [3,11].

Chemicals

ATP, ouabain (strophanthin-G), β-glycerophosphate, glucose 6-phosphate, AMP, potassium ferricyanide, potassium fluoride, deoxycholate, EGTA, EDTA and Dowex 50W-X8 resin were purchased from the Sigma Chemical Company, St. Louis, MO, U.S.A.; furosemide was generously provided by Medicamentos York (Caracas); ^{24}Na (as $NaNO_3$) was provided by Reactor Venezolano I, IVIC, Caracas.

Results

Effect of Ca^{2+} on the ouabain-insensitive Na^+ uptake

Table I shows the effect of 50 μM Ca^{2+} on the Mg^{2+} + ATP-dependent Na^+ uptake in inside-out basolateral plasma membrane vesicles. The vesicles were preincubated at 0°C for 2 h in the presence of Na^+ and ATP or Na^+ and ATP and Ca^{2+}. The suspensions were warmed to 37°C and Mg^{2+} or water were added to start the experiment. The Mg^{2+} + ATP-dependent, Na^+ uptake was calculated as the difference in the Na^+ uptake of vesicles incubated in the presence or absence of Mg^{2+}. It may be seen in Table I that the vesicles show an ouabain insensitive and Mg^{2+} + ATP-dependent Na^+ uptake (b − a) that is clearly enhanced by the addition of Ca^{2+} to the assay medium (c − a).

Effect of Ca^{2+} on the concomitant ATP hydrolysis associated with the Na^+ uptake

Parallel experiments were done for the determination of the concomitant ATP hydrolysis associated with the Mg^{2+} + ATP-dependent Na^+ uptake. The vesicles were preincubated and incubated in the same way as for the Na^+ uptake experiments but without ^{24}Na. The determination of the ATP hydrolysis not associated with the Na^+ uptake (Mg^{2+}-ATPase) was measured incubating the vesicles in a medium without Na^+ and with 2 mM furosemide to avoid any expression of the Na^+-ATPase [3,11]. The results are shown in Table II. The ATP hydrolysis associated with the Na^+ uptake was calculated as the difference between the values obtained in the presence of Mg^{2+} + ATP + Na^+ (c) and in the presence of Mg^{2+} + ATP + furosemide (a). The effect of Ca^{2+} on the ATP hydrolysis associated with the Na^+ uptake was studied in the presence of 50 μM Ca^{2+} in the assay medium for both Mg^{2+} (b) and (Mg^{2+} +

TABLE I

EFFECT OF Ca^{2+} ON THE OUABAIN-INSENSITIVE Na^+ UPTAKE IN INSIDE-OUT BASOLATERAL PLASMA MEMBRANE VESICLES

The vesicles were prepared with 2 mM ouabain. The experiments were performed at pH 7.2 in the presence of 2 mM ATP, 50 mM NaCl and 2 mM ouabain, with or without 5 mM Mg^{2+}. The free Ca^{2+} concentration in the assay medium was 50 μM. All the uptake values were corrected for the nonspecific binding of Na^+ to the membranes [11]. The values are expressed as the mean ± S.E. ($n = 10$).

Incubation medium	Na^+ uptake (nmol Na^+/mg protein per min)
a. Na^+ + ATP	32 ± 3
b. Na^+ + ATP + Mg^{2+}	52 ± 4
c. Na^+ + ATP + Mg^{2+} + Ca^{2+}	70 ± 3
d. (b − a)	20 ± 5 [a]
e. (c − a)	38 ± 5 [a]

[a] $P < 0.001$.

Na^+)-ATPase (d) activities. Notice that the Mg^{2+}-ATPase activity is the same in the presence or absence of 50 μM Ca^{2+} (b − a), which is an indication that the vesicles do not exhibit Mg^{2+}-dependent Ca^{2+}-ATPase activity under these conditions. When the vesicles are transporting Na^+, there is a concomitant ATP hydrolysis as indicated by the difference between the (Mg^{2+} + Na^+)-ATPase and the Mg^{2+}-ATPase (c − a). The effect of Ca^{2+} on this concomitant ATP hydrolysis is indicated as (d − b). It can be seen in this table that Ca^{2+} enhances the ATP hydrolysis associated with the Na^+ uptake. In other words, Ca^{2+} is able to stimulate the Na^+ uptake as well as the concomitant ATP hydrolysis

Effect of furosemide on the active Na^+ uptake and the concomitant ATP hydrolysis

In a previous study [11] we were able to show that 2 mM furosemide totally inhibits the ouabain-insensitive, Na^+ uptake and its concomitant ATP hydrolysis. If the Ca^{2+} effect on the Na^+ uptake and on the ATP hydrolysis is really associated with the ouabain-insensitive Na^+-pump and not with another system, furosemide must be able to inhibit the Ca^{2+} effect. Table III shows the effect of 2 mM furosemide on the ouabain-insensitive, Na^+ uptake and its concomitant ATP hydrolysis in the presence or absence of 50 μM Ca^{2+} in the assay medium. It is clear that furosemide is able to inhibit all the Na^+ uptake and its concomitant ATP hydrolysis, even in the presence of Ca^{2+}.

Is the Ca^{2+} stimulation of the Na^+ uptake due to the operation of the Na^+-Ca^{2+} exchange system?

The existence of an Na^+-Ca^{2+} exchange system has been demonstrated in isolated basolateral plasma membranes from rat kidney cortex [15,16]. If this system is working in our preparations, the effect of Ca^{2+} on the Na^+ uptake could occur as follows: the addition of Ca^{2+} to the incubation medium leads to an increase in Ca^{2+} inside the vesicles (e.g., via a Ca^{2+}-pump). The Ca^{2+} inside the vesicles is exchanged for Na^+, in this way increasing the intravesicular Na^+. The activity of the Na^+-Ca^{2+} exchange system in our preparation was tested in two ways. First, if the Na^+-Ca^{2+} exchange system is operating, we could expect that the Na^+ uptake in the absence of Mg^{2+}, i.e., under conditions where the Ca^{2+}-pump is not working, would be reduced when the vesicles are incubated

TABLE II

EFFECT OF Ca^{2+} ON THE ATP HYDROLYZED BY THE VESICLES DURING THE Na^+ UPTAKE

The vesicles were incubated at 37°C for 1 min, in the same media utilized to study Na^+ uptake. ATP concentration 2 mM. Free Ca^{2+} in the assay medium was 50 μM. Parallel experiments were run without adding Na^+ to the incubation medium and in the presence of 2 mM furosemide, to determine the Mg^{2+}-ATPase. The values are expressed as the mean ± S.E. (n = 10). In this and in the following table, the differences for the ATP hydrolysis were calculated and analyzed using paired data.

Incubation medium	ATPase activity (nmol P_i/mg protein per min)
a. Mg^{2+} + ATP + furosemide	317 ± 6
b. Mg^{2+} + ATP + Ca^{2+} + furosemide	318 ± 6
c. Mg^{2+} + ATP + Na^+	333 ± 7
d. Mg^{2+} + ATP + Na^+ + Ca^{2+}	348 ± 8
(b − a)	1 ± 1
(c − a)	16 ± 2 [a]
(d − b)	30 ± 3 [a]

[a] $P < 0.001$.

TABLE III

EFFECT OF 2 mM FUROSEMIDE ON THE Na^+ UPTAKE AND ITS CONCOMITANT ATP HYDROLYSIS

The Na^+ uptake and the ATP hydrolysis experiments were carried out as indicated under Materials and Methods. Ca^{2+} concentration 50 μM, ATP 2 mM, Mg^{2+} 5 mM, Na^+ 50 mM. The Na^+ uptake values were obtained as the differences in Na^+ uptake in the presence and absence of Mg^{2+} (for details see Table I). The ATP hydrolysis values were obtained as the differences in ATP hydrolysis in the presence of Mg^{2+} + Na^+ and in the presence of Mg^{2+} + furosemide (for details see Table II). The values are expressed as the mean ± S.E. (n = 10).

Incubation conditions	(nmols/mg protein per min)	
	Na^+ uptake	ATP hydrolysis
Control	19 ± 4 [a]	14 ± 3 [a]
Furosemide	3 ± 4	1 ± 4
Ca^{2+}	40 ± 5 [a]	26 ± 4 [a]
Ca^{2+} + furosemide	2 ± 4	5 ± 4

[a] $P < 0.001$.

with increasing concentrations of Ca^{2+}. This is not the case, the Mg^{2+}-independent Na^+ uptake is not affected by increasing concentrations of Ca^{2+}, up to 1 mM (data not shown). This is an indication that the Na^+-Ca^{2+} exchange system is not working, at least exchanging intravesicular Na^+ by Ca^{2+} from the medium. Second, perhaps the system is asymmetric and requires increase in the internal Ca^{2+} due to the Ca^{2+}-pump. Even when we found no evidence for the activity of the (Mg^{2+} + Ca^{2+})-ATPase in our preparations (Table II), we could not exclude the activity of the Ca^{2+}-pump. Dissipation of the putative Ca^{2+} gradient by the Ca^{2+}-ionophore A23187 would inactivate the Na^+-Ca^{2+} exchange. The addition of the ionophore A23187, does not affect the active Na^+ uptake either in the absence (21 ± 5, control, and 20 ± 3 nmol/mg protein per min, with ionophore, $n = 10$) or in the presence (43 ± 6, control, and 40 ± 5 nmol/mg protein per min, with ionophore, $n = 10$) of Ca^{2+}. In view of these results, we do not feel that the effect of Ca^{2+} on the Na^+ uptake is due to the operation of an Na^+-Ca^{2+} exchange system.

Effect of EGTA on the ouabain-insensitive Na^+ uptake

In a previous paper [10], we have found that if the basolateral plasma membranes are treated with 2 mM EGTA for 3–4 h at 0°C, the subsequently measured ouabain-insensitive Na^+-ATPase is inactivated. This inactivation can be reversed by the presence of 50 μM Ca^{2+} in the assay medium. In order to test the effect of this treatment on the active Na^+ uptake, we preincubated the vesicles with 2 mM EGTA for 4 h at 0°C. After the treatment, the vesicles were washed to remove EGTA and the active Na^+ uptake was measured by incubating the vesicles with Na^+ + ATP in the presence or absence of Mg^{2+} (for details see under Materials and Methods). The active Na^+ uptake was inhibited by the EGTA treatment (2 ± 5 nmol/mg protein per min, $n = 10$). The active Na^+ uptake, however, was maximal when the assay was performed in the presence of 50 μM Ca^{2+} in the incubation medium (39 ± 4 nmol/mg protein per min, $n = 10$). This Ca^{2+}-dependent Na^+ uptake was totally blocked by 2 mM furosemide.

Since with Ca^{2+} in the assay medium, the active Na^+ uptake is always maximal, it is important to study the effect of fast removal of Ca^{2+} in vesicles not treated with EGTA. The Na^+ uptake was assayed in the presence of 50 μM Ca^{2+}. After 30 s of incubation, excess of EGTA (0.5 mM) was added to some of the tubes in order to chelate all the Ca^{2+}, while some tubes were left with Ca^{2+}. Samples were taken at 30, 60 and 90 s after the beginning of the incubation. Fig. 1 shows the results of this experiment. The active Na^+ uptake assayed in the presence of Ca^{2+} increased linearly with time with a slope of 38 nmol/mg protein per min. Upon addition of excess of EGTA, the rate of Na^+ uptake decreased immediately to 19 nmol/mg protein per min and resembles the slope of the Na^+ uptake determined in the absence of Ca^{2+}. Addition of furosemide to the incubation medium under these conditions abolished completely the active Na^+ uptake (data not shown).

Na^+ uptake as a function of free Ca^{2+}

To examine further the effect of Ca^{2+} on the active Na^+ uptake by the vesicles not treated with

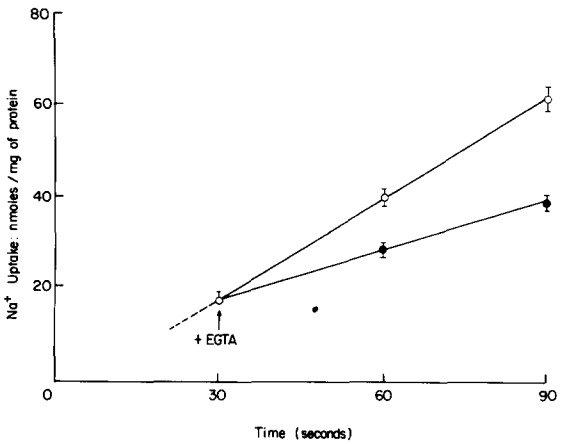

Fig. 1. Time-course of Na^+ uptake of basolateral plasma membrane vesicles in the presence or absence of Ca^{2+}. The vesicles were preincubated for 2 h at 0°C and then incubated for different lengths of time in the following medium (mM): NaCl 50 with 50 μCi/ml ^{24}Na; Tris-HCl (pH 7.2) 10; ATP 2; $MgCl_2$ 5; $CaCl_2$ 0.05; sucrose, in enough quantity to obtain an osmolarity of 300 mosmol/l. The open circles correspond to experiments carried out with Ca^{2+} in the assay medium; filled circles to experiments carried out after addition of 500 μM EGTA to the assay medium. The values of the slopes were 38 (with Ca^{2+}) and 19 nmol/mg protein per min (in the presence of EGTA). The cumulative values for Na^+ uptake are expressed as the mean ± S.E. ($n = 10$).

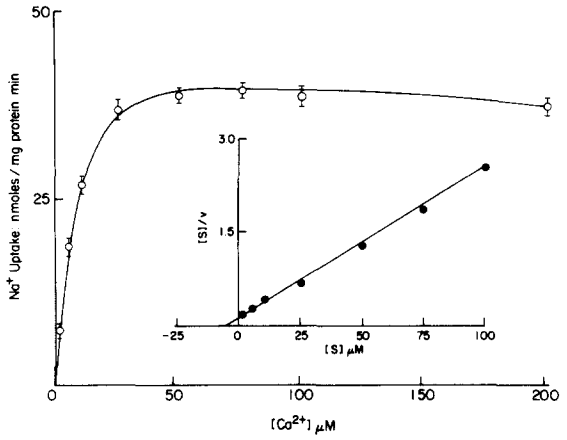

Fig. 2. Effect of different free Ca^{2+} concentrations on the Na^+ uptake by basolateral plasma membrane vesicles. The vesicles were preincubated for 2 h at 0°C and then incubated in the same media utilized to study Na^+ uptake with different concentrations of free Ca^{2+}. The desired free Ca^{2+} concentrations were calculated from the multiple equilibria involving ATP, EGTA, Ca^{2+} and Mg^{2+} [16]. The values are expressed as the mean ± S.E. ($n = 10$). The apparent K_a was calculated by means of the Woolf derivative of the Lineweaver-Burk transformation of the Michaelis-Menten equation, of the form:

$$S/V = K_a/V_{max} + S/V_{max}$$

where $K_a = -S$ on the S axis (see inset).

EGTA, additional experiments were carried out where the Na^+ uptake was assessed at different free Ca^{2+} concentrations. In these experiments we preset the free Ca^{2+} concentrations, which were calculated from the multiple equilibria involving ATP, EGTA, Ca^{2+} and Mg^{2+} [16]. Fig. 2 shows the Ca^{2+} enhanced Na^+ uptake, as a function of free Ca^{2+} concentration. The free Ca^{2+} concentration was varied from 1 to 200 μM. The optimal Ca^{2+} concentration is around 25 μM and the apparent K_a, calculated by the Woolf variation of the Lineweaver-Burk transformation of the Michaelis-Menten equation with the data of Fig. 2, as shown in the inset, is around 5 μM.

Discussion

The main purpose of this work was to evaluate the effect of Ca^{2+} on the magnitude of ouabain-insensitive Na^+ uptake in inside-out basolateral plasma membrane vesicles prepared from rat kidney proximal tubular cells. The principal findings of this study are: (1) micromolar concentrations of Ca^{2+} in the assay medium enhance the ouabain insensitive Na^+ uptake (Table I) and the ATP hydrolysis associated with this Na^+ uptake (Table II); (2) both Na^+ uptake and concomitant ATP hydrolysis are inhibited by 2 mM furosemide (Table III); (3) the Ca^{2+} effect is not the result of the activity of a Na^+-Ca^{2+} exchange system; (4) long periods of treatment of the vesicles with 2 mM EGTA inhibit the ouabain-insensitive Na^+ uptake and this effect can be reversed by the presence of Ca^{2+} in the assay medium; (5) maximal Na^+ uptake (in the presence of Ca^{2+}) is inhibited by about 50% when Ca^{2+} is removed by the presence of excess of EGTA in the incubation medium (Fig. 1); and (6) the ouabain-insensitive Na^+ uptake shows an apparent K_a for free Ca^{2+} of around 5 μM (Fig. 2).

The observations summarized above indicate that Ca^{2+} can modulate the activity of the ouabain-insensitive Na^+-pump. The modulation of the Na^+-pump is accomplished possibly by the interaction of Ca^{2+} with the basolateral plasma membranes of the proximal tubular cells. This interaction is made through two different ways that depend on how high is the affinity of Ca^{2+} for the membranes. (1) Loosely bound Ca^{2+} as a labile component, which can be quickly and easily 'deactivated' by a large reduction of the free Ca^{2+} concentration (Fig. 1). (2) Tightly bound Ca^{2+} as a stable component, which can be 'deactivated' by 3–4 h of preincubation of the membranes with large concentrations of EGTA. Ca^{2+} can very easily activate both components.

The effect of Ca^{2+} on the ouabain-insensitive Na^+ uptake and its concomitant ATP hydrolysis (Tables I and II) could be explained as due to a disaggregating effect of Ca^{2+} on the vesicle populations. In other words, when we assay either the Na^+ uptake or the ATP hydrolysis there exist different populations of vesicles that are aggregated. Therefore, the Na^+ uptake and its concomitant ATP hydrolysis could be reduced because the access to the sites of one or more of the ligands becomes restricted or impossible. When we add Ca^{2+} to the assay medium, the population of vesicles are disaggregated, the access to the sites is increased and therefore the activity of the Na^+-pump is also increased. This is not the case, since

we have found, working with the same preparation but treated with deoxycholate + EDTA (broken membranes), that the ouabain-insensitive Na^+-ATPase is enhanced by micromolar concentrations of Ca^{2+} with no changes in its affinity for Na^+ [10].

It is difficult at present to provide a straightforward explanation for the activating effect of Ca^{2+}. Perhaps this effect of Ca^{2+} is either on a membrane-bound regulatory protein, e.g. calmodulin or as we suggested in our previous paper [10] might be related to some conformational change produced by the interaction of Ca^{2+} with the membranes, since the ouabain-insensitive Na^+-ATPase is also maximized by resuspending the membranes at pH 7.8 [1] or by ageing the preparations [9,10]. Under the last two circumstances, Ca^{2+} does not exert any additional effect on the Na^+-ATPase [10].

The finding that the active Na^+ uptake into the vesicles is not a result of a Na^+-Ca^{2+} exchange could be taken as an indication that the ouabain-insensitive Na^+ extrusion in the proximal tubular cell is accomplished mainly by an Na^+-pump and not by the activity of the Na^+-Ca^{2+} exchange mechanism, secondary to the function of the Ca^{2+}-pump. The modulation of the Na^+-pump by Ca^{2+} might have an important physiological significance as this pump could be involved in cell volume regulation. When the intracellular free Ca^{2+} concentration is low, the Na^+ extrusion accompanied by Cl^- and water is also low. This low Na^+ extrusion is still, however, modulated by some Ca^{2+} bound to the basolateral plasma membrane. When there is a rise in the intracellular free Ca^{2+} concentration above 5 μM (Fig. 2) the extrusion of NaCl and water is increased. Now the Na^+-pump is modulated both by the Ca^{2+} already bound to the membrane and by a new Ca^{2+} bound to the membrane due to the increased cytoplasmic free Ca^{2+}. In this way, an increase in the intracellular free Ca^{2+} concentration would promote an enhancement in the cell water extrusion across the basolateral plasma membrane of the renal proximal tubular cells. As soon as the intracellular free Ca^{2+} concentration returns to normal values, the new Ca^{2+} bound to the membrane is released, the activity of the Na^+-pump is reduced and, therefore, the cell water extrusion is concomitantly reduced.

It is still subject to debate whether this ouabain-insensitive Na^+-pump does exist separately from the classical Na^+/K^+-pump or not. However, several facts must be considered: (1) furosemide inhibits 100% the K^+-independent Na^+ uptake and its concomitant ATP hydrolysis, without producing any effect on the K^+-dependent Na^+ uptake and ATP hydrolysis (see Table II and III, Ref. 11); (2) ouabain, even under conditions where the Na^+/K^+-pump is practically 100% inhibited, does not produce any effect on the K^+-independent Na^+ uptake and its concomitant ATP hydrolysis. In the absence of K^+, these values are the same either in the presence or absence of ouabain (for details see Ref. 11); (3) the Na^+/K^+-pump is inhibited by micromolar concentrations of intracellular Ca^{2+} [17–21], while the same concentrations of Ca^{2+} elicit the maximal activity of the ouabain-insensitive Na^+-pump (Table I). These experimental facts can be explained considering the existence of two different Na^+-pumps rather than the existence of the Na^+/K^+-pump as the only mechanism responsible of the cellular Na^+ extrusion. The results presented in this work provide strong support for the existence of a furosemide-sensitive Na^+-pump, which is modulated by Ca^{2+}, in the basolateral plasma membranes of renal proximal tubular cells.

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