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OUABAIN-INSENSITIVE Na^+-STIMULATED ATPase ACTIVITY OF BASOLATERAL PLASMA MEMBRANES FROM GUINEA-PIG KIDNEY CORTEX CELLS

II. EFFECT OF Ca^{2+}

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The ouabain-insensitive, Mg^{2+}-dependent, Na^+-stimulated ATPase activity present in fresh basolateral plasma membranes from guinea-pig kidney cortex cells (prepared at pH 7.2) can be increased by the addition of micromolar concentrations of Ca^{2+} to the assay medium. The Ca^{2+} involved in this effect seems to be associated with the membranes in two different ways: as a labile component, which can be quickly and easily 'deactivated' by reducing the free Ca^{2+} concentration of the assay medium to values lower than 1 μM; and as a stable component, which can be 'deactivated' by preincubating the membranes for periods of 3–4 h with 2 mM EDTA or EGTA. Both components are easily activated by micromolar concentrations of Ca^{2+}. The K_a of the system for Na^+ is the same, 8 mM, whether only the stable component or both components, stable and labile, are working. In other words, the activating effect of Ca^{2+} on the Na^+-stimulated ATPase is on the V_{max}, and not on the K_a of the system for Na^+. The activating effect of Ca^{2+} may be related to some conformational change produced by the interaction of this ion with the membranes, since it can also be obtained by resuspending the membranes at pH 7.8 or by ageing the preparations. Changes in the Ca^{2+} concentration may modulate the ouabain-insensitive, Na^+-stimulated ATPase activity. This modulation could regulate the magnitude of the extrusion of Na^+ accompanied by Cl^- and water that these cells show, and to which the Na^+-ATPase has been associated as being responsible for the energy supply of this mode of Na^+ extrusion.

Introduction

Two Na^+-stimulated ATPase activities have been demonstrated in basolateral plasma membranes from guinea-pig kidney proximal tubular cells. One is the ouabain-sensitive $(Na^+ + K^+)$-ATPase, which is only partially inhibited by ethacrynic acid. The other, is the ouabain-insensitive Na^+-ATPase, which is totally inhibited by ethacrynic acid [1]. These ATPase activities correlate with the observation that cells of guinea-pig kidney cortex slices (rich in proximal tubules), when rewarmed in a medium with K^+, after leaching in the cold in a medium without K^+ (to load them with Na^+), extrude Na^+ actively in two different modes. In mode A, Na^+ is extruded in exchange for K^+. In mode B, Na^+ is extruded along with Cl^- and water. Mode A is ouabain-sensitive and is only partially inhibited by ethacrynic acid. Mode B is ouabain-insensitive and is totally inhibited by ethacrynic acid [2,3]. In view of the

Abbreviation: EGTA, Ethyleneglycolbis(aminoethyl ether)-*N,N'*-tetraacetic acid.

parallelism between the characteristics of the two Na^+-stimulated ATPase activities and the two modes of Na^+ extrusion, it has been proposed that each of these two ATPases is responsible for the energy supply of each of these two modes of active Na^+ movement across the basolateral plasma membranes out of the proximal tubular cells [1].

Recent evidence supports further the relation between the two modes of Na^+ transport and the two ATPase activities described above. Thus, working with inside-out basolateral plasma membrane vesicles prepared from rat kidney cortex cells (rich in proximal tubules) we have described the presence of two different Na^+ movements across the vesicle membranes and of two ATPase activities associated with them. One is seen when Na^+ and K^+ are added to the incubation medium containing Mg^{2+} plus ATP. It is specifically inhibited by ouabain. The other is seen, even in the presence of ouabain, when only Na^+ is added to the same incubation medium. It is specifically inhibited by ethacrynic acid [4,5].

Several approaches have been utilized to demonstrate the ouabain-insensitive, Na^+-ATPase activity: (1) ageing of microsomal preparations at 4°C [6]; (2) resuspension of basolateral plasma membrane preparations at pH 7.8 [1]; and (3) measurement of the ATPase activity in the presence of micromolar quantities of Ca^{2+} [1].

The present work was undertaken to study the activating effect of Ca^{2+} on the Na^+-ATPase activity of the basolateral plasma membranes prepared from guinea-pig kidney cortex slices. The results suggest that, possibly through a conformational change, Ca^{2+} modulates the activity of the Na^+-ATPase. Ca^{2+} exerts its effects by altering the V_{max} of the Na^+-ATPase, without affecting the K_a of the system for Na^+.

Materials and Methods

Preparation of fractions enriched in basolateral plasma membranes

Outermost slices of kidney cortex (which are known to be rich in proximal tubules) of healthy adult guinea-pig (*Cavia porcellus*) were obtained as already described [2]. The slices were homogenized at 4°C in 3 vol. 250 mM sucrose/20 mM Tris-HCl buffer (pH 7.2) with eight strokes at 2500 rpm using an Eberbach homogenizer with a Teflon pestle. The homogenate was centrifuged as previously described [1]. The treatment of the tissue included an important modification as compared with our previous method, namely, elimination of EDTA from the media used. The final pellet was resuspended in the described sucrose/Tris medium at pH 7.2 and frozen and kept at −20°C until use. Any modification of the resuspending medium is indicated for the specific experiment. The preincubations with EDTA or EGTA were carried out at 0°C in the same 250 mM sucrose/20 mM Tris-HCl medium to which 2 mM EDTA or EGTA was added. Before the enzymatic assays, the membranes were treated with deoxycholate and EDTA according to the method of Jørgensen and Skou [7]. When the membranes were preincubated with EDTA or EGTA, the required amount of deoxycholate (final concentration 0.06% for pH 7.2 and 0.1% for pH 7.8) was added 30 min before the end of the preincubation period and the treatment was followed at 23°C. The purity of the fraction was tested by the determination of different enzymatic markers as described before [1].

Assay of the Na^+-stimulated ATPase activity

The method already described was followed [1]. Briefly, required amounts of the membrane suspensions (25 µl) were preincubated for 5 min at 37°C and then incubated for 15 min in the presence of (final concentrations, mM): 50–150 Tris-HCl (pH 6.9) 5 $MgCl_2$, 1 ouabain and, when required, 100 NaCl, 0.025–0.10 $CaCl_2$, 0.1 EGTA. The final volume was 1 ml. The reaction was started by adding Na^+-free ATP (2 mM final concentration) and stopped by the addition of 1 ml of ice-cold 6% $HClO_4$. The samples were chilled, centrifuged and the liberated orthophosphate (P_i) was determined in the deproteinized solution [8]. All samples were run in triplicate. The protein content of the original suspensions was measured by the folin method [9]. The ATPase activity is expressed as nmol P_i liberated per mg protein per min, after subtraction of a blank run in parallel without the membrane suspension, which was added after the $HClO_4$. The Na^+-stimulated ATPase activity was calculated as the difference between the values obtained when the assays were performed in the presence or absence of Na^+. The

use of 1 mM ouabain completely inhibits any $(Na^+ + K^+)$-ATPase activity present in the preparation [1].

Source of materials

Na^+-free, vanadate-free ATP; ouabain (Strophanthin-G), EDTA, EGTA and deoxycholate, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Results

The free Ca^{2+} concentration in our preparations without any treatment, measured with an Orion electrode, was 28 ± 2 μM ($n = 6$). Therefore, during preincubation with 2 mM EDTA or EGTA, the free Ca^{2+} concentration must be less than $1 \cdot 10^{-9}$ M. In addition, during the ATPase assays the free Ca^{2+} is diluted 40-times (25 μl preparation in 1 ml final volume). Therefore Ca^{2+} concentration can be considered much less than 10^{-9} M under these conditions in the assay media.

Similarly for the EDTA or EGTA concentration in the ATPase assay media, the preincubation media in all cases contained 2 mM of either EDTA or EGTA. Since these media are diluted 40-times for the ATPase assay, the EDTA or EGTA concentration in the ATPase assay media becomes 50 μM. These concentrations of chelators were taken into account when Ca^{2+} was added to the ATPase assay media. When EDTA was used, all the EDTA was chelated by the 5 mM Mg^{2+} present in the assay medium, and Ca^{2+} was added to a concentration of 50 μM. When the preincubations were done with EGTA, Ca^{2+} was added to the ATPase assay medium to reach a concentration of 50 μM.

Fig. 1 shows the effect on the Na^+-ATPase activity of preincubating the membranes at 0°C with either 2 mM EDTA or EGTA during times ranging from 0 to 4 h. 30 min before the end of the preincubation periods, the membranes were warmed up to 23°C and 0.06% deoxycholate (final concentration) was added to the media.

It may be seen in Fig. 1 that the membranes preincubated with EDTA or EGTA for periods of up to 2 h show the already described Na^+-ATPase activity [1]. But this activity decreases and even disappears if the membranes are preincubated with

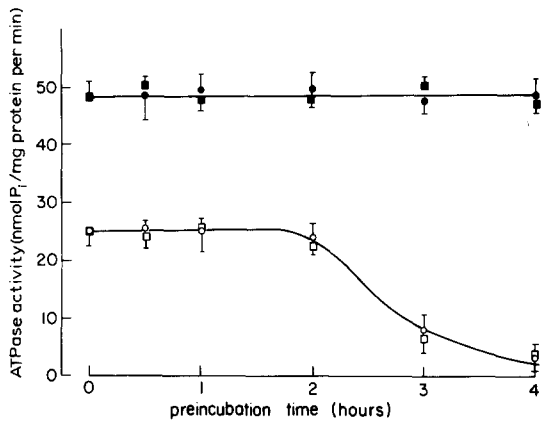

Fig. 1. Effect of the addition of Ca^{2+} to the assay medium on the Na^+-ATPase activity of membranes preincubated for different lengths of time with 2 mM EDTA (circles) or EGTA (squares). The zero-time samples were in contact with the chelating agents for about 30 s. At the end of the preincubation times the samples were assayed for ATPase activity. Open symbols correspond to the assays carried out in the absence of Ca^{2+} and filled symbols correspond to the assays carried out in the presence of Ca^{2+}. The Ca^{2+} added to the assay medium was 50 μM for membranes treated with EDTA and 100 μM for membranes treated with EGTA. The experiments were carried out in the presence of 2 mM ATP, 5 mM Mg^{2+} and 1 mM ouabain. The Na^+ concentration was 100 mM. The Na^+-ATPase activity was calculated as the difference between the activity in the presence of $Mg^{2+} + Na^+$ minus the activity in the presence of Mg^{2+}. The values are expressed as the mean ± S.E. ($n = 10$).

EDTA or EGTA for period longer than 2 h. In addition, Fig. 1 clearly shows that the Na^+-ATPase activity is stimulated by Ca^{2+}. When the ATPase assays are run in the presence of 50 μM Ca^{2+}, the Na^+-ATPase activity is always optimal and the inhibitory effect of long preincubations with EDTA or EGTA disappears. This establishes a difference from the $(Na^+ + K^+)$-ATPase activity, which is partially inhibited by the presence of 50 μM Ca^{2+} [1].

The higher Na^+-ATPase activity observed in the presence of Ca^{2+} could be due either to an activating effect of this ion on the Na^+-ATPase or to the expression of a Ca^{2+}-ATPase activity. Even when the latter possibility had already been ruled out [1], several experiments were performed to study the effect of Ca^{2+} on the Na^+-ATPase activity. Table I shows the Na^+-ATPase activity of freshly prepared membranes and of membranes

TABLE I

EFFECT OF THE PRESENCE OF 50 μM Ca^{2+} IN THE ASSAY MEDIUM ON THE Na^+-STIMULATED ATPase ACTIVITY

Freshly prepared and 12 days aged basolateral plasma membranes were resuspended at pH 7.2 or 7.8 and were preincubated for 30 min with 2 mM EDTA (control) or for 4 h with 2 mM EDTA or EGTA. The assays were performed at pH 6.9 in the presence of 5 mM Mg^{2+}, 2 mM ATP and 1 mM ouabain. The Na^+ concentration was 100 mM. The free Ca^{2+} concentration in the incubation medium, when this ion was no added, was lower than $1 \cdot 10^{-10}$ M The values are expressed as the mean ± S.E. ($n = 10$).

pH of resuspension	Preincubation conditions	Na^+-ATPase activity (nmol P_i/mg protein per min)			
		Fresh membranes		Aged membranes	
		$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$
7.2	Control	24 ± 3	55 ± 6	29 ± 3	32 ± 4
7.8		57 ± 3	55 ± 5	32 ± 4	36 ± 1
7.2	2 mM EDTA (4 h)	5 ± 3	52 ± 5	34 ± 3	32 ± 2
7.8		53 ± 5	54 ± 3	30 ± 2	30 ± 3
7.2	2 mM EGTA (4 h)	6 ± 2	49 ± 3	30 ± 4	32 ± 3
7.8		51 ± 3	52 ± 3	30 ± 5	32 ± 2

aged for 12 days exposed to different conditions. After the last centrifugation, the membranes were resuspended at pH 7.2 or 7.8. In both cases, a part of the membrane fractions was immediately used (fresh membranes) or aged in a refrigerator at 4°C for 12 days (aged membranes). In all the cases, before the incubations, the membranes were preincubated at 23°C for 30 min with 2 mM EDTA (control) or for 4 h with 2 mM EDTA or EGTA. The fresh membranes resuspended at pH 7.2 showed the same behavior as that shown in Fig. 1. When the assays were performed in the absence of Ca^{2+}, the control fresh membranes showed an Na^+-ATPase activity of 24 ± 3 nmol P_i mg protein per min. On the other hand, the membranes treated for 4 h with 2 mM EDTA or EGTA did not show any Na^+-ATPase activity. The presence of 50 μM Ca^{2+} in the assay medium elicited maximal activity under all conditions. When the membranes were resuspended at pH 7.8 and preincubated for 30 min (control) or for 4 h with 2 mM EDTA or EGTA, they showed maximal Na^+-ATPase activity in the absence or in the presence of 50 μM Ca^{2+} in the assay medium. On the other hand, under all conditions tested, the aged membranes showed similar Na^+-ATPase activity (ranging from 29 ± 3 to 34 ± 3 nmol P_i/mg protein per min). It must be noticed that this activity is lower than the maximal activity of the fresh membranes. This is due to the fact that the ageing process results in a progressive diminution of the ATPase activity.

The above results clearly indicate that the Na^+-stimulated ATPase can be activated by methods other than addition of Ca^{2+}, i.e., by resuspending the membranes at pH 7.8 or by ageing the preparations at 4°C. Control experiments were carried out in the presence of the Na^+ ionophore gramicidin (6.25 μg/mg protein). The results were the same as for the experiments performed in the absence of the ionophore, indicating that the lower values of the Na^+-ATPase obtained under several conditions could not be explained as due to the formation of vesicles.

Since in the presence of Ca^{2+} in the assay medium, the Na^+-ATPase activity is always maximal, it is important to study, in each case, the time-course of the activating effect of Ca^{2+}. Fig. 2 shows the time-course of the Na^+-ATPase activity of membranes resuspended at pH 7.2, preincubated for 30 min with 2 mM EDTA and assayed in a medium without Ca^{2+}. After 10 min, 50 μM Ca^{2+} was added to some of the tubes, while some were left without Ca^{2+}. The ATPase activity determined in the absence of Ca^{2+} (control) increased linearly with time with a slope of 28 nmol P_i/mg protein per min. Upon addition of Ca^{2+}, the ATPase activity increased immediately, also linearly with time, the slope being 57 nmol

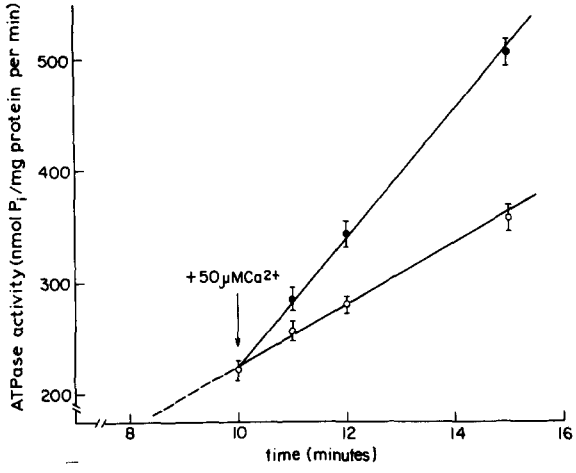

Fig. 2. Time-course of Na^+-ATPase activity of fresh membranes resuspended at pH 7.2 and preincubated for 30 min with 2 mM EDTA. The open circles correspond to an experiment carried out without the addition of Ca^{2+} to the assay medium, filled circles to that carried out after addition of 50 μM Ca^{2+} to the assay medium. The values of the slopes were 28 (without Ca^{2+}) and 57 nmol P_i/mg protein per min (in the presence of Ca^{2+}). The cumulative values for P_i formation are expressed as the mean ± S.E. ($n = 10$).

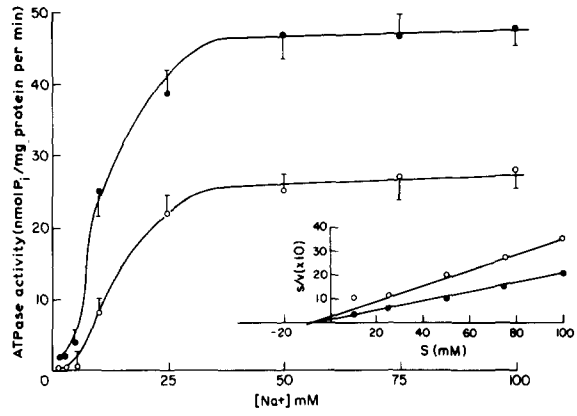

Fig. 3. Effect of Na^+ concentration on the Na^+-ATPase activity of fresh membranes resuspended at pH 7.2 and preincubated for 30 min with 2 mM EDTA. Experiments were carried out in the absence (control, open circles) or in the presence of 50 μM Ca^{2+} (filled circles) in the assay medium. The values 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).

P_i/mg protein per min. Membranes resuspended at pH 7.8 show slopes of 55 nmol P_i/mg protein per min, in the absence or in the presence of 50 μM Ca^{2+}. If the same experiment is carried out with membranes resuspended at pH 7.2 and preincubated for 4 h with 2 mM EGTA, the Na^+-ATPase activity is linearly raised by the presence of Ca^{2+}, from a value near zero (slope = 2 nmol P_i/mg protein per min) to a value of 53 nmol P_i/mg protein min, similar to that shown in Fig. 2 for the Ca^{2+}-activated slope. The time-course of the ATPase activity, determined in the presence of 50 μM Ca^{2+} (control), with a slope of 50 nmol P_i/mg protein per min was immediately decreased upon addition of 100 μM EGTA, linearly, with a slope of 26 nmol P_i/mg protein per min. Similar experiments performed with membranes resuspended at pH 7.8 showed an Na^+-ATPase activity of 55 nmol P_i/mg protein per min, and addition of 100 μM EGTA to the incubation medium did not change this activity.

Fig. 3 shows the activating effect of 50 μM Ca^{2+} in the incubation medium at different Na^+ concentrations on the Na^+-ATPase activity of membranes resuspended at pH 7.2 and preincubated for 30 min with 2 mM EDTA. Notice that in the presence or absence of Ca^{2+} (control) the optimal Na^+ concentration is around 40 mM and about 8 mM is the apparent K_a, calculated by the Woolf variation of the Lineweaver-Burk equation, (insert of Fig. 3). The V_{max}, on the other hand, was raised by the presence of Ca^{2+} from a value of near 29 nmol P_i/mg protein per min to a value of 48 nmol P_i/mg protein per min. Similar values of K_a (8 mM) and V_{max} (50 nmol P_i/mg protein per min) were obtained when membranes resuspended at pH 7.8 and preincubated for 4 h with 2 mM EDTA or EGTA were assayed for Na^+-ATPase activity in the presence or absence of 50 μM Ca^{2+}.

Discussion

The present results indicate that Ca^{2+} can modulate the activity of the ouabain-insensitive, Na^+-ATPase associated to basolateral plasma membranes from guinea-pig kidney cortex cells (in

preparations resuspended at pH 7.2), possibly by interacting with these membranes. The Ca^{2+} involved in the modulation of the Na^+-ATPase, seems to be associated to the membranes in two different ways: as a labile component, which can be quickly and easily 'deactivated'; and as a stable component, which can be 'deactivated' by 3 to 4 h of preincubation of the membranes with large concentrations (2 mM) of EDTA or EGTA (Fig. 1). Ca^{2+} can very easily activate both components.

It is difficult at present to provide a straightforward explanation for the activating effect of Ca^{2+}. It seems related to some conformational change produced by the interaction of this ion with the membranes, since it can also be obtained by other methods, i.e., by resuspending the membranes at pH 7.8 or by ageing the preparations (Table I). Under the last two circumstances, stable Na^+-ATPase activity values are obtained in the absence of Ca^{2+} and this ion does not exert any effect on the activity of the Na^+-ATPase.

The intracellular concentration of ionized calcium in most cells is exceedingly low, with values at or below $1 \cdot 10^{-6}$ M [10–13]. It is well known that Ca^{2+} plays a critical role as a factor coupling several cellular processes like excitation to contraction [14] and stimulus to secretion [15–19]. A transient increase in the ionized calcium in the cytosol following a stimulus is the trigger for these physiological mechanisms.

It has been considered [1,4,5] that the Na^+-ATPase present in our membrane preparations is responsible for the energy supply for the extrusion of Na^+ accompanied by Cl^- and water, a mechanism demonstrated to exist in the cells from which these membranes are prepared. Ca^{2+} could modulate this extrusion of Na^+ as follows. At a very low (intracellular) Ca^{2+} concentration, the system would work at a low rate (only the stable component being 'activated'). A rise in the intracellular Ca^{2+} concentration would 'activate' the labile component and the Na^+ transporting system would start working at a higher rate (since stable and labile components would be 'activated'). As soon as the intracellular Ca^{2+} concentration returns to normal values, the re-established normal Ca^{2+} gradient will 'deactivate' the labile component, and the system will return to work at its normal lower rate.

Both components, stable and labile, show equal affinity for Na^+ (Fig. 3, see inset), indicating that the modulating effect of Ca^{2+} is exerted on the speed at which the process takes place. At the moment we are determining the K_a for Ca^{2+} of both components.

The fact that the Na^+-ATPase activity can be increased by μM concentrations of Ca^{2+}, which, on the other hand, produces a partial inhibition of the $(Na^+ + K^+)$ATPase activity [1], together with the fact that the kidney cortex slices extrude more Na^+ accompanied by Cl^- and water and less Na^+ interchanged by K^+ when incubated in a medium with a high (5 mM) Ca^{2+} concentration [20], may be taken as an indication of a possible role of Ca^{2+} in the control of the way these cells handle their Na^+ content. Further investigations must be realized to determine the physiological implications of the present observations.

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