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# Inside-out basolateral plasma membrane vesicles from rat kidney proximal tubular cells

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**A method for preparation of highly purified basolateral plasma membranes from rat kidney proximal tubular cells is reported. These membranes were assayed for the presence of vesicles as well as for their orientation. $(Na^+ + K^+)$-ATPase activity and [$^3$H]ouabain binding studies with membranes treated with or without SDS revealed that the preparation consisted of almost 100% vesicles. The percentage of inside-out vesicles was found to be approx. 70%. This percentage was determined measuring the $(Na^+ + K^+)$-ATPase activity in $K^+$-loaded vesicles and in membranes treated with or without trypsin and SDS. These membranes represent a very efficient tool to assay the correlation between active transport and ATPase activities in basolateral plasma membranes from rat kidney proximal tubular cells.**

## Introduction

Since the activity of the $(Na^+ + K^+)$-ATPase was correlated to the activity of the $Na^+,K^+$-pump [1], a great amount of work has been done in order to demonstrate the correlation between the mechanisms responsible for active transport of ions through the membranes with the activity of different ATPases [2–8]. Plasma membranes usually form vesicles, and this characteristic has been used by several authors to demonstrate transport mediated by the activity of the $(Na^+ + K^+)$-ATPase [7,9,10]. This implies the use of different techniques in order to investigate the sites of the ligands, due to asymmetry of the enzyme. $(Na^+ + K^+)$-ATPase requires $Mg^{2+}$, $Na^+$ and ATP at the cytoplasmic face of the cell membrane, and it requires $K^+$ and is inhibited by ouabain at the extracellular face of the membranes.

Renal tissue is one of the richest sources of $(Na^+ + K^+)$-ATPase [11], and membrane preparations from mammalian kidney have been utilized to study transport mediated by $(Na^+ + K^+)$-ATPase [6,9,10]. These preparations consist of a mixed population of vesicles with different orientations [6,7,9,10,12]. Right-side-out vesicles have been assayed for transport using osmotic shock in order to put ATP and $Mg^{2+}$ into the vesicles [6]. However, this technique has limitations: (1) the real concentrations of the ligands in the vesicles are unknown; (2) the drastic treatment can produce changes in the membrane structure or permeability; (3) long-term experiments cannot be performed. The ideal preparation is represented by inside-out vesicles, which eliminate the limitations of the right-side-out vesicle preparations. However, there is, as yet, no known technique for preparing purified basolateral plasma membrane fractions from kidney tissue enriched in inside-out

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Abbreviation: PMSF, phenylmethylsulfonyl fluoride.

vesicles. The amount of inside-out vesicles, for different preparations reported, is not higher than 20% of the total population of vesicles [6,7,9,11]. Although this percentage is somewhat low, these preparations have been used for transport studies, by preincubating the vesicles with $K^+$ and then adding $Mg^{2+}$, $Na^+$ and ATP to the incubation medium [9]. In a recent paper [10], we reported the preparation of basolateral plasma membranes from cortical cells of rat kidney with 70% inside-out vesicles from a total population consisting of 90% vesicles and 10% membrane sheets. However, this preparation was poorly enriched in ($Na^+ + K^+$)-ATPase (around 5-times higher than the specific activity in homogenate). In the present work, a different preparation of basolateral plasma membranes from kidney cortex cells, with a higher degree of purification of the ($Na^+ + K^+$)-ATPase, was assayed for inside-out vesicles.

It was found that the percentage of inside-out vesicles, determined by different approaches, is approx. 70% from a total of almost 100% vesicles.

## Materials and Methods

*Preparation of fractions enriched in ($Na^+ + K^+$)-ATPase*

Healthy male Sprague-Dawley rats (3 months old) were anesthetized with diethyl ether and then immediately killed by decapitation. The kidneys were removed, decapsulated and collected in a medium containing 250 mM sucrose/20 mM Tris-HCl (pH 7.2)/0.5 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride (medium 1), at 4°C. The outermost slices of the kidney cortex (which are rich in proximal tubules) were homogenized and centrifuged at 4°C using a modification of the method of Kinsella et al. [13]. The slices were homogenized at 4°C, with eight strokes at 2500 rev/min in an Eberbach homogenizer with a tight-fitting Teflon pestle, in 3 vol. medium 1 per g tissue. The homogenate (usually 240 mg protein) was centrifuged at $1000 \times g$ for 10 min and the supernatant was saved. The pellet was resuspended with medium 1 and recentrifuged at $1000 \times g$ for 10 min. The resulting supernatant was combined with the previous supernatant. This fraction was centrifuged at $9500 \times g$ for 10 min. The supernatant and the soft, lighter upper portion of the pellet were combined and centrifuged at $48\,000 \times g$ for 20 min. The soft, lighter upper portion of the pellet was resuspended with a solution of 25 mM Tris-HCl (pH 7.2)/100 mM mannitol/2 mM $CaCl_2$/1 mM $MgCl_2$/1 mM $MnCl_2$ (medium 2) to a final protein concentration of 4–6 mg/ml. This membrane suspension was kept on ice for 90 min and centrifuged at $1400 \times g$ for 12 min. The pellet was resuspended with the medium 2 and reextracted again at the same speed. The final pellet was resuspended in 25 mM Tris-HCl (pH 7.2)/100 mM mannitol to a final protein concentration of 2–4 mg/ml. A 5-ml aliquot of this final suspension was layered over a discontinuous sucrose gradient consisting of 10 ml 893 mM sucrose and 10 ml 250 mM sucrose/20 mM Tris-HCl (pH 7.2) (medium 3). This gradient was centrifuged at $48\,000 \times g$ for 20 min. At the end of the centrifugation period, the sucrose gradient was carefully removed by aspiration and the soft, lighter upper portion of the pellet was resuspended with medium 3. This final suspension was dialyzed for 30 min at 4°C against 100 vol. medium 3 and re-dialyzed overnight at 4°C against 100 vol. of the same solution. At the end of the dialysis period, this final basolateral plasma membrane preparation was assayed for ($Na^+ + K^+$)-ATPase activity. In previous experiments, the basolateral plasma membrane preparation treated with SDS was assayed for the presence of other cellular membranes, i.e. luminal, mitochondrial, lysosomal and endoplasmic reticulum. It was found that the activities of the enzymatic markers associated to these membranes were significantly lower than those in the homogenate (data not shown). This is an indication of the purity of the basolateral plasma membrane fraction.

*ATPase assays*

The ($Na^+ + K^+$)-ATPase activity (EC 3.6.1.37) was measured by a modification of a previously described method [14,15]. A 180-$\mu$l aliquot of the incubation medium, containing (final concentrations) 50 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 2 mM Tris-ATP and when required 7 mM ouabain, was preincubated at 37°C for 5 min. The reaction was started by adding 20 $\mu$l of the membrane suspension (0.1 mg protein/ml) and continued until the indicated

time. The reaction was stopped and inorganic phosphate was determined following the method of Baginski et al. [16], modified by Ottolenghi [17], Brotherus et al. [18], Forbush [19] and ourselves. At the end of the incubation period, 300 μl of an ice-cold solution of 2.8% ascorbic acid/0.48 M HCl/0.48% ammonium molybdate/2.8% SDS were added and the test tubes were placed on ice. After 10 min at 0°C, 500 μl of a solution of 2% sodium arsenite/2% sodium citrate/2% acetic acid were added and the test tubes were rewarmed to 37°C for 10 min. At the end of the rewarming period the absorbance was determined at 705 nm ($16 \pm 2$ $A/\mu$mol $P_i$ in the above volume). All samples were run in quadruplicate. The activity was expressed as nmol of $P_i$ liberated per mg protein per min, after subtraction of a blank run in parallel without the membrane suspension, which was added after the ascorbic acid/HCl/ammonium molybdate/SDS solution. The protein content of the original suspension was determined by the Coomassie blue method [20,21]. $(Na^+ + K^+)$-ATPase activity was calculated as the difference between the $P_i$ liberated in control tubes and that liberated in the presence of 7 mM ouabain.

*SDS pretreatment*

250 μl of membrane suspension (0.1 mg protein/ml) were treated with different amounts of SDS, in the presence of 1% bovine serum albumin/25 mM imidazole (pH 7.0)/2 mM EDTA. After incubation for 20 min at 37°C, the SDS-treated membranes were assayed for $(Na^+ + K^+)$-ATPase activity as described above. Control membranes were treated in the same way but without SDS. SDS was the detergent chosen to open the vesicles present in the membrane suspensions because it has been found to be more effective than any other detergent [19].

*Other assays*

The specific [$^3$H]ouabain binding was carried out with membranes treated with or without SDS, following the method developed by Tobin and Sen for renal membranes [22].

The trypsin pretreatment of the membranes was performed according to the method already described by Forbush [6].

*Chemicals*

ATP, ouabain (strophanthin-G), porcine trypsin (T-0134, nominally 10000 units/mg), soybean trypsin inhibitor, bovine serum albumin, EDTA, PMSF and dithiothreitol were purchased from the Sigma Chemical Company; sodium dodecyl sulfate (SDS) was obtained from Bio-Rad Laboratories; [$^3$H]ouabain and Instagel were purchased from New England Nuclear.

Statistical analysis was performed by Student's *t*-test. All results are expressed as means ± S.E. and ($n$) represents the number of experiments performed with different membrane preparations.

## Results

*Determination of vesicles*

Fig. 1 shows the effect of SDS treatment on $(Na^+ + K^+)$-ATPase activity of our purified basolateral plasma membrane fraction. It can be seen that the activity is increased with the treatment, reaching an optimal value at an SDS/protein ratio of 1.6. The highest value obtained for $(Na^+ + K^+)$-ATPase activity is $1974 \pm 19$ nmol $P_i$/mg protein per min, which represents an en-

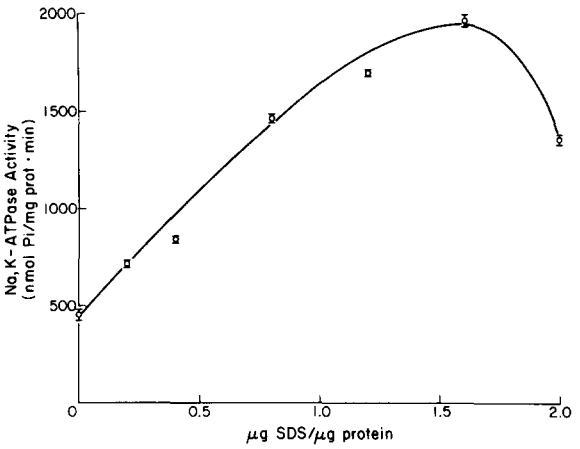


Fig. 1. Effect of the treatment of basolateral plasma membranes with SDS on the activity of the $(Na^+ + K^+)$-ATPase. At the end of the treatment, 2 mM EDTA was added to the treatment medium. The ATPase assay was carried out in the following medium (mM): NaCl, 100; KCl, 20; $MgCl_2$, 5; Tris-ATP, 2, and Tris-HCl, 50. The $(Na^+ + K^+)$-ATPase activity was determined from the difference in $P_i$ released in control tubes and in the presence of 7 mM ouabain. Values are expressed as means ± S.E. ($n = 6$).

richment of approx. 33-fold when compared with the activity of homogenates ($60 \pm 3$ nmol $P_i$/mg protein per min, in homogenates treated with SDS at the optimal SDS/protein ratio). The yields of protein and $(Na^+ + K^+)$-ATPase activity in the purified fraction are $0.42 \pm 0.02\%$ and $14 \pm 1\%$, respectively. The enrichment found indicates a purification of basolateral plasma membranes, since the $(Na^+ + K^+)$-ATPase has been demonstrated to be a specific marker for these membranes [24,25]. Detergent activation of the $(Na^+ + K^+)$-ATPase is the result of rupturing of the tight vesicles or increased permeability of the vesicles to the different ligands. The results presented in Fig. 1 also show that the activity of the $(Na^+ + K^+)$-ATPase in the absence of any treatment is approx. 25% of the maximal activity. This 25% activity is due to broken or leaky vesicles, since tight vesicles prevent access to one or more of the ligands of the $(Na^+ + K^+)$-ATPase. Accordingly, we can estimate that this fraction consists of at least 75% of tight vesicles, regardless of their orientation. The broken or leaky vesicles can be due to spontaneous rupture of vesicles during the assay, since this is performed for 10 min at 37°C. If this is true, and the rupture or increased leakiness is irreversible, the $(Na^+ + K^+)$-ATPase activity in membranes without any treatment would be higher in experiments carried out at longer than at shorter times. To study this point, the ouabain-sensitive $(Na^+ + K^+)$-ATPase activity of basolateral plasma membrane vesicles was measured for different incubation times (Table I). It should be noted that the activity is higher for longer incubation times, reaching maximal values between 5 and 10 min of incubation. It is also very important to note that during the first few seconds of incubation, there is practically no ouabain-sensitive $(Na^+ + K^+)$-ATPase activity, indicating the absence of broken or leaky vesicles. However, ouabain could not be fully inhibiting the $(Na^+ + K^+)$-ATPase activity during most of the 0.1 min incubation. In this case, the activity in the presence and absence of ouabain would be very similar, and the difference (ouabain-sensitive activity) would be very small. This is not the case, since a similar experiment, performed with a preparation preincubated with ouabain for 5 min at 37°C (with no $Mg^{2+}$, which was added to start the ATPase activity), gave

TABLE I

$(Na^+ + K^+)$-ATPase ACTIVITY OF BASOLATERAL PLASMA MEMBRANE VESICLES MEASURED AT DIFFERENT LENGTHS OF INCUBATION TIME

The activity is the result of the difference in $P_i$ liberated in the medium containing $Mg^{2+} + Na^+ + K^+ + ATP$ in the absence or presence of 7 mM ouabain. Values are expressed as means ± S.E. ($n = 6$).

| Incubation time (min) | $(Na^+ + K^+)$-ATPase activity (nmol $P_i$/mg protein per min) |
|---|---|
| 0.1 | $32 \pm 15$ |
| 1 | $104 \pm 19$ |
| 2 | $385 \pm 16$ |
| 5 | $536 \pm 18$ |
| 10 | $538 \pm 23$ |

results similar to those mentioned in Table I (data not shown). These results can be explained by assuming that the purified basolateral membrane fraction consists almost entirely of vesicles, some of which may be broken during the $(Na^+ + K^+)$-ATPase assay.

Another way to assess the amount of vesicles present in the preparation is by performing studies of [$^3$H]ouabain binding. Ouabain binds to the extracellular face of plasma membranes only when appropriate ligands are present at the opposite face. [$^3$H]Ouabain binding is supported by either $Mg^{2+} + Na^+ + ATP$ or $Mg^{2+} + P_i$ [6,22,23]. Accordingly, if [$^3$H]ouabain binding is carried out in the presence of $Mg^{2+} + Na^+ + ATP$, ouabain cannot bind to the sealed vesicles, regardless of their orientation, because it cannot reach its site in the inside-out vesicles, and ATP cannot reach its site in the right-side-out vesicles. If the vesicles are ruptured with SDS treatment, the ligands can reach their sites to support [$^3$H]ouabain binding and this should be maximal. This experiment was performed and the results show that the [$^3$H]ouabain bound to control membranes represents only 5% of the [$^3$H]ouabain bound to membranes treated with SDS ($16 \pm 4$ compared to $318 \pm 20$ pmol/mg protein, respectively). The small quantity of ouabain bound to control membranes may be due to the presence of membrane sheets or vesicles leaky to ouabain in the plasma membrane preparation. These results are in complete agreement with those determined by measuring the

activity of the $(Na^+ + K^+)$-ATPase in membranes without any treatment, at different incubation times (Table I).

*Estimation of orientation of basolateral plasma membrane vesicles*

Since during the first few seconds of incubation we still have 100% tightly sealed vesicles, we studied their orientation by assaying their $(Na^+ + K^+)$-ATPase activity during a short period of time (2 s) under different incubation conditions. The vesicles were pre-loaded with 20 mM KCl for 24 h at 4°C. The $K^+$-loaded vesicles treated with or without SDS were assayed for $(Na^+ + K^+)$-ATPase activity (Table II). As a furosemide-sensitive $Na^+$-ATPase has been described for these membranes [10,14,15], all the experiments were performed in the presence of 2 mM furosemide, which completely inhibits the $Na^+$-ATPase without any effect on the $(Na^+ + K^+)$-ATPase (the activity of the $Na^+$-ATPase is studied in the following paper [27]). In Table II, it can be seen that there is an apparent ouabain-insensitive $(Na^+ + K^+)$-ATPase activity in $K^+$-loaded vesicles not treated with SDS (b − a, no treatment). This activity is similar to that obtained in the absence of ouabain (c − a, no treatment). When the vesicles are treated with SDS, the apparent ouabain-insensitive $(Na^+ + K^+)$-ATPase activity disappears (b − a, SDS treatment), whereas the $(Na^+ + K^+)$-ATPase activity in the absence of ouabain is maximal and similar to the maximal value presented in Fig. 1 (c − a, SDS treatment). These results indicate the presence of inside-out vesicles impermeable to ouabain in our preparations, which can be estimated to amount to 72%.

The estimation of 72% inside-out vesicles in the purified basolateral plasma membrane fraction can be confirmed in additional experiments by treating the membranes with trypsin. This proteinase has been demonstrated to eliminate the activity of the $(Na^+ + K^+)$-ATPase [26]. Trypsin cleaves $(Na^+ + K^+)$-ATPase from the cytoplasmic face, but not from the extracellular face of the plasma membranes [6,26]. These experimental facts are useful to determine the presence and the quantity of right-side-out vesicles, since they do not expose the cytoplasmic face of the plasma membrane. If we treat the membranes with trypsin, only the $(Na^+ + K^+)$-ATPase activity in broken, leaky and inside-out vesicles would be inactivated. The tryptic digestion of the $(Na^+ + K^+)$-ATPase was carried out by incubating the vesicle preparations with trypsin for 15 min. At the end of this incubation, the trypsin inhibitor was added to stop the reaction. As shown in Fig. 1, the $Na^+,K^+$-ATPase activity is maximal in membranes treated with SDS ($1982 \pm 24$ nmol $P_i$/mg protein per min). When the membranes were treated with SDS and then with trypsin, the $(Na^+ + K^+)$-ATPase was completely inactivated ($6 \pm 2$ nmol $P_i$/mg protein per min). This is an indication that the concentration of trypsin utilized is able to inactivate all the $(Na^+ + K^+)$-ATPase activity. When the membranes were treated with trypsin and then with SDS, the activity of the $(Na^+ + K^+)$-ATPase was decreased by approx. 70% ($687 \pm 18$ nmol $P_i$/mg protein per min). This indicates that approx. 70% of the vesicles had the cytoplasmic portion of the $(Na^+ + K^+)$-ATPase exposed (inside-out vesicles), whereas approx. 30% of vesicles had the trypsin-sensitive face of the ATPase inaccessible to trypsin (right-side-out vesicles). Membranes treated by inverting the order of treatment, by addition of

TABLE II

$(Na^+ + K^+)$-ATPase ACTIVITY IN $K^+$-LOADED VESICLES TREATED OR NOT WITH SDS AND ASSAYED FOR 2 s

The vesicles were preincubated for 24 h at 4°C with 20 mM KCl. 10 min before the ATPase assay the suspensions were rewarmed to 37°C. Each assay was carried out in sextuplicate (each tube was run separately). All the assays were performed in the presence of 5 mM $MgCl_2$/2 mM Tris-ATP/2 mM furosemide. The concentration of ouabain utilized was 7 mM. The SDS treatment was carried out using the optimal SDS/protein ratio as indicated in Fig. 1. The $(Na^+ + K^+)$-ATPase activity was calculated as the difference between the total activity (rows b or c) and the Mg-ATPase activity (row a). Values are expressed as means ± S.E. ($n = 7$), in nmol $P_i$/mg protein per min.

| Additions | Treatment | |
|---|---|---|
| | none | SDS |
| a. $K^+$ + ouabain | 404 ± 21 | 30 ± 17 |
| b. $Na^+ + K^+$ + ouabain | 1812 ± 77 | 28 ± 21 |
| c. $Na^+ + K^+$ | 1866 ± 57 | 2004 ± 48 |
| b − a | 1408 ± 80 | −2 ± 27 |
| c − a | 1462 ± 61 | 1976 ± 52 |

trypsin inhibitor, trypsin and then SDS, again show maximal activity of the $(Na^+ + K^+)$-ATPase ($2006 \pm 18$ nmol $P_i$/mg protein per min).

## Discussion

In this work, we report the preparation of basolateral plasma membrane enriched fractions from rat kidney proximal tubular cells (33-fold enrichment in $(Na^+ + K^+)$-ATPase activity when compared with crude homogenate). This fraction was tested for the presence of vesicles using different techniques: $(Na^+ + K^+)$-ATPase activity (Table II) and [$^3$H]ouabain binding in membranes treated with or without SDS. Using these methods, we have demonstrated that the membrane fraction consists almost entirely of vesicles. Taking advantage of the asymmetry of the $(Na^+ + K^+)$-ATPase, we prepared $K^+$-loaded vesicles and the activity of the $(Na^+ + K^+)$-ATPase was measured. This resulted in a preparation containing 70% of the maximal activity even in the presence of ouabain (Table II). This 70% represents the percentage of inside-out vesicles present in the purified basolateral plasma membrane fraction. The confirmation of this value was achieved by tryptic digestion of the $(Na^+ + K^+)$-ATPase. Trypsin only destroys the $(Na^+ + K^+)$-ATPase activity when the cytoplasmic face of the plasma membrane is accessible. Our results confirm the percentage of inside-out vesicles (70%) present in the membrane fraction. The 30% difference is represented by right-side-out vesicles and some broken or leaky vesicles. The fact that the percentage of inside-out vesicles is similar when determined by two different techniques ($K^+$-loaded vesicles and tryptic digestion) argues against the possibility that the vesicles are, in the first case, leaky to $Mg^{2+}$, $Na^+$ and ATP (in order to explain the results presented in Table II) or leaky to trypsin. Furthermore, if the vesicles are leaky to $Mg^{2+}$, $Na^+$ and ATP, how can we explain the fact that the [$^3$H]ouabain binding in vesicles not treated with SDS is only a very small percentage (5%) of the maximal binding?. The only explanation is that the membranes are tightly vesiculated.

It is important to mention that the method reported in this paper for preparation of inside-out vesicles is not necessarily general for all basolateral plasma membranes from mammalian kidney cortex cells. Actually, the original method reported by Kinsella et al. [13] for dog kidney cortex plasma membranes produced mostly (77%) right-side-out vesicles. Furthermore, other authors [7,9], using different techniques for the isolation of basolateral plasma membranes from rabbit or rat kidney cortex, have reported the presence of a low percentage of inside-out vesicles.

Finally, it is necessary to discuss the importance of a purified plasma membrane fraction enriched in inside-out vesicles. Most of the studies of correlation between ATPase activity and active transport performed using natural membrane vesicles have been carried out with very small amounts of transporting vesicles. In the case of inside-out vesicles, the main problem is the small percentage of these vesicles in the preparations [7,9]. On the other hand, in preparations enriched in right-side-out vesicles [6], apparently only 10% of the vesicles can perform transport functions due to limitations of the osmotic shock technique. However, despite the small percentages of transporting vesicles, elegant studies have been performed correlating transport and ATPase activities [6,7]. The use of this preparation enriched in inside-out vesicles (70%) for transport studies represents a very efficient tool to ascertain the role of ATPases in active transport processes through the basolateral plasma membrane of rat kidney proximal tubular cells.

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