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ATPase ACTIVITIES IN KIDNEY BASOLATERAL PLASMA MEMBRANES OF YOUNG AND OLD RATS

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Abstract—The present work studied the turnover rate of $(Na^+ + K^+)$-ATPase as well as Mg^{2+}- and Na^+-ATPase activities in basolateral plasma membranes from kidney cortex cells of young and old rats. It was found that, as for the homogenates, the turnover rate of the $(Na^+ + K^+)$-ATPase was diminished by aging in about 40%. The Mg^{2+}-ATPase activities on the other hand, were similar for the rat kidneys of young and old, in both the homogenates as well as the basolateral plasma membrane fractions.

In a recent paper [1], we have shown that ouabain-insensitive, Na^+-stimulated ATPase and ouabain-sensitive, $(Na^+ + K^+)$-stimulated ATPase of homogenates from rat kidney cortex slices (rich in proximal tubules) are diminished by aging. Even though these results partially corroborate the findings of Beauchene *et al.* [2], regarding the diminution of the $(Na^+ + K^+)$-ATPase of rat renal tissue with aging, it must be considered that any increment of nonspecific proteins in the tissue from the old rats would diminish the expression of the ATPase activities, giving the results shown.

To test this possibility, it was decided to study the ATPase activities and the [^{3}H]ouabain binding of basolateral plasma membrane-enriched fractions of kidney cortex slices from old (24 months) and young (3 months) rats. It was found that, as for the homogenates, the ratio $(Na^+ + K^+)$-ATPase/[^{3}H]ouabain binding was lower for the old than for the young rats. This may be taken as an indication that the turnover rate of the $(Na^+ + K^+)$-ATPase was diminished in the kidneys of old rats.

MATERIALS AND METHODS

Isolation of basolateral plasma membranes. The experiments were done with young (3 months) and old (24 months) male Sprague–Dawley rats. Outermost kidney slices (0.2 to 0.3 mm in thickness) were obtained as described before [3]. The slices were homogenized and the homogenate was purified as already described [4]. Briefly, 1 g of tissue was homogenized in 3 ml of medium containing 250 mM sucrose and 20 mM Tris–HCl (pH 7.2). The homogenization was performed at 4° with eight strokes at 2500 rpm in an Eberbach homogenizer with a Teflon pestle. The homogenate (H) (usually 240 mg protein) was filtered through two sheets of gauze to remove clumps and spun at 1475 *g* for 10 min; the pellet was resuspended in three vol. of 2 M sucrose and rehomogenized with two strokes in the same homogenizer used before. The supernatant fraction was discarded. The resuspension was spun at 13,300 *g* for 10 min. The pellet was discarded, and the supernatant fraction was taken back to isotonicity with 7 vol. of cold distilled water and then spun at 35,000 *g* for 15 min. The supernatant fraction and the lower pellet (brown layer, rich in mitochondria) were discarded, and the upper peilet (pink layer) was resuspended in sucrose–Tris medium and spun for 1 min at 48,000 *g*. The pellet (P_{3c}) was resuspended in the same medium, to give about 2 mg protein/ml of final suspension. The material so prepared was kept in the freezer at −20°. Before the enzymatic assays, the fractions were treated with 0.06% deoxycholate (DOC) and 2 mM EDTA, for 30 min at room temperature, following the method of Jørgensen and Skou [5], to avoid vesicle formation.

Assay of the ATPase activity (EC 3.6.1.3*).* Aliquots of the different fractions (0.1 to 0.2 mg protein) were preincubated for 5 min at 37° in the presence of (final concentrations): 50–150 mM Tris–HCl (pH 7.0), 5 mM $MgCl_2$ and, when required, 100 mM NaCl, 20 mM KCl, 50 μM $CaCl_2$ and 2 mM ouabain. The osmolarity of the media was adjusted by varying the concentration of Tris. The reaction was started by adding to the medium Na^+-free, Tris-ATP (2 mM final concentration). The final volume was 1 ml. After 15–30 min, the incubation was terminated by the addition of 1 ml of ice-cold 6% $HClO_4$ to the incubation tubes. The samples were chilled and centrifuged. The liberated phosphate was determined in the deproteinized solution [6]. The protein content of the original suspensions was measured by the method of Lowry *et al.* [7]. All samples were run in triplicate or quadruplicate. ATPase activity is expressed as nmoles of phosphorus produced per mg of protein per min of incubation, after subtraction of a blank run in parallel without the aliquots of membrane suspension, which were added only after the addition of the $HClO_4$. The Mg^{2+}-ATPase activity refers to the ATPase activity measured in the presence of Mg^{2+} alone in the incubation medium. Na^+-ATPase and $(Na^+ + K^+)$-ATPase activities refer to the differences between

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$(Mg^{2+} + Na^+)$-ATPase and Mg^{2+}-ATPase activities and between $(Mg^{2+} + Na^+ + K^+)$-ATPase and $(Mg^{2+} + Na^+)$-ATPase activities respectively. The Na^+-ATPase activity was determined in the presence of 50 μM Ca^{2+}, as described previously [8, 9]. Considering the small values of the Na^+-ATPase activity, the quantity of protein and the time of incubation, for each experiment, were carefully chosen in order to obtain significant differences.

Other enzymatic assays. The following methods were used: acid phosphatase (EC 3.1.3.2) was determined using β-glycerophosphate at pH 5.4 as substrate [10]; 5′-nucleotidase (EC 3.1.3.5) was determined with 5′-AMP as substrate at pH 8.5 [11]; glucose-6-phosphatase (EC 3.1.3.9) was determined with glucose-6-phosphate as substrate and with 4 mM EDTA and 2 mM potassium fluoride, at pH 6.7, to inhibit the nonspecific phosphatases [10]; and succinate dehydrogenase (EC 1.3.99.1) was determined following the succinate-dependent reduction of K^+-ferricyanide according to the method of King [12].

[^{3}H]ouabain binding. Following the method of Tobin and Sen [13], ouabain binding was carried out as follows: protein (2 mg), previously treated with DOC + EDTA, was incubated for different lengths of time and with different ouabain concentrations at 37° in 10 ml of a medium containing (mM): imidazole–HCl (pH 7.4), 20; NaCl, 120; ATP, 2; and $MgCl_2$, 2. The specific activity of the [^{3}H]ouabain was 0.66 mCi/ml. At the end of the incubation time, the tubes were put on ice and immediately centrifuged at 48,000 *g* for 17 min at 0°. The pellet was washed once in the same medium without [^{3}H] ouabain and then solubilized with 500 μl of Triton X-100 at 10%. Instagel (4.5 ml) was added to the solubilized solution, and the radioactivity was determined. The binding of [^{3}H]ouabain is expressed as pmoles [^{3}H]ouabain per mg of protein, after subtracting the nonspecific binding, determined following the same method but without adding ATP to the incubation medium. ATP is essential for the specific incorporation of ouabain under these conditions [13]. The nonspecific binding of ouabain represented, in all the cases, 1–3% of the specific binding obtained under optimal conditions. These results are in close agreement with those of Tobin and Sen [13].

Source of materials. β-Glycerophosphate, glucose-6-phosphate, ATP, AMP, ouabain (Strophanthin-G), K^+-ferricyanide, potassium fluoride, EDTA and deoxycholate were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. [^{3}H]Ouabain was purchased from the New England Nuclear Corp., Boston, MA, U.S.A.

RESULTS

Table 1 shows the ATPase activities and the [^{3}H] ouabain binding in kidney cortex homogenates from young and old rats. The $(Na^+ + K^+)$-ATPase activity was nearly 40% lower for the old rats, whereas the specific [^{3}H]-ouabain binding was similar for the two groups of animals. Accordingly, the ratio "$(Na^+ + K^+)$-ATPase activity/[^{3}H]ouabain binding" was nearly 40% lower for the old rats. The Na^+-ATPase activity was also lower (nearly 40%) for the old rats, whereas the Mg^+-ATPase activity was similar in both cases. This was not the case for 6-month-old rats, which gave results similar to the 3-month-old rats (data not shown).

The distribution of the tested enzymes, through the different fractions obtained during the purification of the basolateral plasma membranes, was very similar for both groups of rats. The $(Na^+ + K^+)$-ATPase, marker for basolateral plasma membranes [14–16], was preferentially purified and recovered (for young and old rats) in the P_{3c} fractions. A similar distribution was obtained for the Na^+-ATPase, but not for the other enzymes, which were preferentially enriched and recovered in some other fractions. The total recovery for protein and all the tested enzymes was, in all the cases, near 100% (data not shown). Figure 1 shows the enrichment factor ("Enzyme activity in P_{3c} fraction/enzyme activity in homogenate") and the percent recovery of the different enzymes in fraction P_{3c} for young and old rats. As mentioned above, in both cases the $(Na^+ + K^+)$- and the (Na^+)-ATPases were preferentially enriched and recovered in this fraction, which was not the case for the other tested enzymes: acid phosphatase, marker for lysosomes [17]; succinic dehydrogenase, marker for mitochondria [18]; glucose-6-phosphatase, marker for endoplasmic reticulum [17, 19, 20]; and

Table 1. ATPase activities and [^{3}H]ouabain binding in homogenates of kidney cortex slices from young (3 months) and old (24 months) rats*

Age (months)	ATPase activity (nmoles P_i/mg protein/min)			[^{3}H]ouabain binding (pmoles/mg protein)	$(Na^+ + K^+)$-ATPase / [^{3}H]Ouabain binding
	Mg^{2+}	Na^+	$Na^+ + K^+$		
3	228 ± 5	9 ± 1	79 ± 6	11.5 ± 0.9	6.87 ± 0.07
24	219 ± 4	5 ± 1	45 ± 7	10.5 ± 0.4	4.29 ± 0.04
Variation	−9 ± 6†	−4 ± 1.4‡	−34 ± 9§	−1 ± 0.98†	−2.58 ± 0.08‖

* The ATPase activities and the [^{3}H]ouabain binding were carried out as indicated under Material and Methods. For the ouabain binding, the incubation time was 2 min and the ouabain concentration was 6.5×10^{-7} M. Values are expressed as mean ± S.E. of six determinations.

† Not significant.

‡ $0.02 < P < 0.05$.

§ $P < 0.01$.

‖ $P < 0.001$.

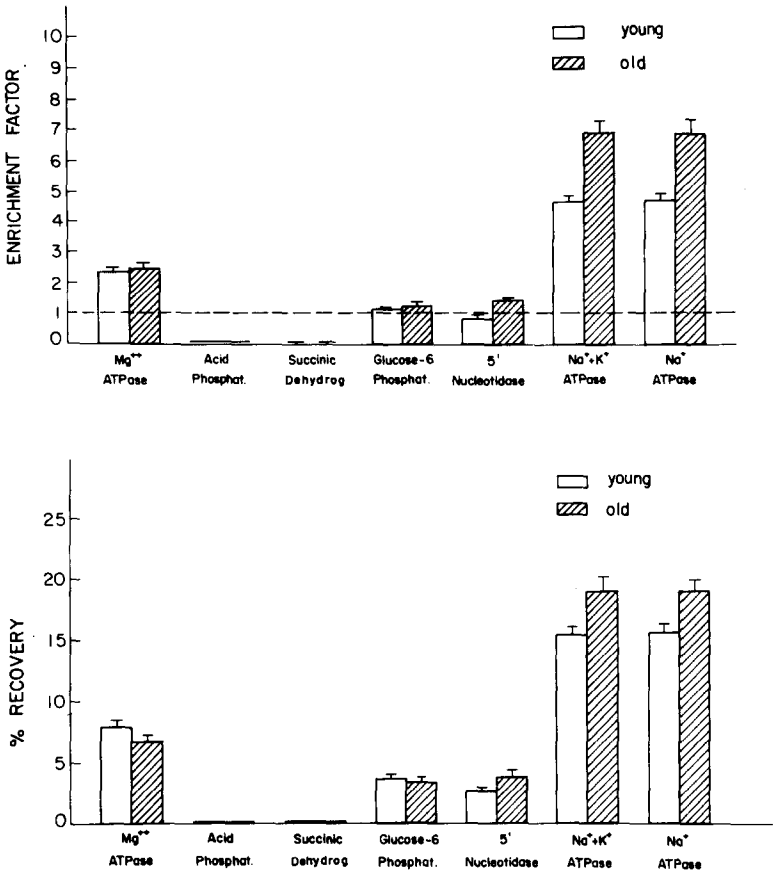

Fig. 1. Enrichment factor (specific activity of each enzyme in fraction P_{3c}/specific activity homogenate) (top panel), and percentage of recovery of the different enzymes in fraction P_{3c} referred to the homogenate (bottom panel), for young and old rats. Values are expressed as mean ± S.E. of ten determinations.

5'-nucleotidase, marker for luminal plasma membranes [18]. The Mg^{2+}-ATPase was not considered, since it is not a specific marker for any particular membrane. These results indicate that the P_{3c} fraction is preferentially enriched in basolateral plasma membranes. The enrichment factor as well as the percent recovery of the $(Na^+ + K^+)$- and the Na^+-ATPase were higher for the old rats (23 and 48% respectively), indicating a higher degree of purification of the basolateral plasma membranes.

Table 2 shows the ATPase activities and the [^{3}H] ouabain binding in P_{3c} fractions from young and old rats. The higher degree of purification of the basolateral plasma membranes obtained for the old rats is reflected in these results. The $(Na^+ + K^+)$- and the Na^+-ATPase activities, which were nearly 40% lower for the homogenate, were similar, whereas the [^{3}H]ouabain binding, the values of which were similar in the homogenates, was nearly 35% higher for the old rats. The ratio "$(Na^+ + K^+)$-

Table 2. ATPase activities and [^{3}H]ouabain binding in basolateral plasma membranes of kidney proximal tubular cells from young (3 months) and old (24 months) rats*

Age (months)	ATPase activity (nmoles P_i/mg protein/min)			[^{3}H]Ouabain binding (pmoles/mg protein)	$(Na^+ + K^+)$-ATPase / [^{3}H]Ouabain binding
	Mg^{2+}	Na^+	$Na^+ + K^+$		
3	408 ± 6	38 ± 2	403 ± 29	60 ± 4	6.72 ± 0.3
24	428 ± 10	39 ± 2	404 ± 32	93 ± 6	4.34 ± 0.2
Variation	+20 ± 12†	+1 ± 3†	+1 ± 43†	+33 ± 7‡	−2.38 ± 0.36‡

* The ATPase activities and the [^{3}H]ouabain binding were carried out as indicated under Materials and Methods. For the ouabain binding, the incubation time was 2 min and the ouabain concentration was 6.5×10^{-7} M. Values are expressed as mean ± S.E. of six determinations.

† Not significant.

‡ $P < 0.001$.

Table 3. [^{3}H]Ouabain binding in basolateral plasma membranes of kidney proximal tubular cells from young (3 months) and old (24 months) rats, as a function of the ouabain concentration*

Ouabain concn (M)	[^{3}H]Ouabain binding (pmoles/mg protein)	
	Young	Old
1.10^{-9}	4 ± 1	5 ± 1
1.10^{-8}	27 ± 1	42 ± 2
1.10^{-7}	58 ± 2	87 ± 2
1.10^{-6}	63 ± 2	90 ± 2
1.10^{-5}	62 ± 2	91 ± 3

* [^{3}H]Ouabain binding was carried out as indicated under Materials and Methods. Incubation time was 4 min. Values are corrected for nonspecific binding (1–3% of the specific binding) and are expressed as mean ± S.E. of six determinations.

ATPase activity/[^{3}H]ouabain binding" for the P_{3c} fraction was lower for the old rats, as were the values obtained for the homogenates.

Even when we measured the specific ouabain binding to the membranes under optimal conditions, according to the method of Tobin and Sen [13] for renal membranes, the results could be explained by our not measuring the maximum specific ouabain binding at equilibrium. To test this possibility, the specific values of ouabain binding to basolateral plasma membranes from young and old rats were evaluated for various ouabain concentrations and lengths of time. The results are shown in Tables 3 and 4. It may be seen in Table 3 that the specific ouabain binding, for both preparations, was almost maximal at a concentration of 1.10^{-7} M ouabain. The time course of the specific ouabain binding was determined using a concentration of 6.5×10^{-7} M ouabain. Table 4 presents the results of this experiment. The specific binding for both preparations was maximal after 30 sec of incubation at 37°. It may be concluded that, under the incubation conditions used in the determinations shown in Tables 1 and 2, the specific ouabain binding for both preparations was optimal and at equilibrium. Consequently, the differences found between the ratios of $(Na^+ + K^+)$-ATPase activity to [^{3}H]ouabain binding for young and old rats cannot be explained as being due to unfavorable incubation conditions.

Table 4. [^{3}H]Ouabain binding in basolateral plasma membranes of kidney proximal tubular cells from young (3 months) and old (24 months) rats, as a function of the incubation time*

Incubation time (sec)	[^{3}H]Ouabain binding (pmoles/mg protein)	
	Young	Old
15	56 ± 2	86 ± 3
30	62 ± 2	90 ± 3
90	64 ± 4	93 ± 5
120	65 ± 5	94 ± 3
240	64 ± 3	94 ± 4

* [^{3}H]Ouabain binding was carried out as indicated under Materials and Methods. The ouabain concentration was 6.5×10^{-7} M.

Values are corrected for nonspecific binding (1–3% of the specific binding) and are expressed as mean ± S.E. of six determinations.

DISCUSSION

The main point of this paper was to determine whether the lowered Na^+- and $(Na^+ + K^+)$-ATPase activities found in homogenates of kidney cortex slices of old rats (Table 1) were real or just due to the presence of nonspecific proteins that could mask the results. This possibility was tested by measuring ATPase activities and [^{3}H]ouabain binding, at equilibrium, of purified basolateral plasma membrane preparations. The ratio "$(Na^+ + K^+)$-ATPase activity/[^{3}H]ouabain binding" for basolateral plasma membrane enriched fractions (Table 2) of old rats was about ⅔ that of young rats, which is similar to the value obtained for the homogenate. If it is considered that: (a) 1 molecule of bound [^{3}H] ouabain is equivalent to 1 $Na^+ + K^+$-pump and (b) 1 molecule of ATP hydrolyzed is equivalent to 1 cycle of the pump [21], the ratio "$(Na^+ + K^+)$-ATPase activity/[^{3}H]ouabain binding" may be used to calculate the turnover of the $Na^+ + K^+$-pump. These values are 6870 ± 70 min^{-1} and 6720 ± 30 min^{-1} for the homogenate and the P_{3c} fraction of the young rats, respectively, and 4290 ± 40 and 4340 ± 20 for the old rats. In both cases, homogenate and basolateral membrane enriched fraction (P_{3c}), the turnover rate of the $Na^+ + K^+$-pump was lower by nearly 38% in the old rats.

If the results obtained for the homogenate were due to the presence of a higher degree of nonspecific proteins in the old rats, this would not be the case for the basolateral plasma membrane purified fraction. It is reasonable to expect that the nonspecific proteins would migrate to some other fraction, in which case the calculated turnover rate for the P_{3c} fraction would be higher or at least different than the one calculated for the homogenate. Since this was not the case, it may be concluded that the turnover rate of the $(Na^+ + K^+)$-ATPase of rat kidney proximal tubular cells is diminished by nearly 40% in the old rats. Similar conclusions cannot be drawn for the Na^+-ATPase, since there is as yet no estimate of the number or density of Na^+ pumps. Much work remains to be done to try to explain the described differences between the old and young rats.

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