Amiloride Directly Inhibits the Na,K–ATPase Activity of Rabbit Kidney Proximal Tubules

Abstract. *Amiloride inhibited the ouabain-sensitive rate of oxygen consumption (QO_2) of a suspension of rabbit intact proximal tubules in the presence of different concentrations of extracellular sodium. Measurements of the ouabain-sensitive QO_2 in the presence of nystatin, the tissue sodium and potassium contents of the tubules in suspension, and the sodium- and potassium-dependent adenosinetriphosphatase (Na,K–ATPase) activity of lysed tubule membranes indicated that the effect of amiloride was due to a direct inhibition of the Na,K–ATPase activity of the proximal tubule.*

Amiloride (*1*) has been used as a specific transport inhibitor in various tissues and is used clinically as a potassium-sparing diuretic. Two general sodium transport mechanisms that are inhibited by amiloride can be roughly distinguished on the basis of the dose of amiloride required to cause the effect (*2*). At low concentrations (< 1 μM), this drug inhibits the sodium entry pathway of the apical cell membranes of tight epithelia (*3*), including those of the toad bladder, rabbit colon, and rabbit renal collecting tubule. At larger concentrations (> 1 μM), another sodium transport pathway, that of Na^+-H^+ exchange, is inhibited in *Necturus* gallbladder (*4*), sea urchin eggs (*5*), and mouse soleus muscle (*6*). The effectiveness of amiloride as a diuretic is due to its action in the kidney; low doses cause natriuresis by blocking the sodium entry pathway of the distal convoluted tubule and collecting duct. Amiloride has also been used in kidney studies as a pharmacological tool to inhibit the Na^+-H^+ exchanger of the proximal tubule. In this capacity, the drug was a reversible competitive inhibitor of the sodium site of the exchanger in proximal microvillus membrane vesicles (*7*) and inhibited H^+ efflux from perfused proximal tubules of rabbit (*8*). However, the inhibitory effect on Na^+-H^+ exchange requires high concentrations of amiloride, and pharmacological drug doses may produce effects that are different from the specific interactions occurring at lower concentrations. We report that when amiloride is used at high concentrations in a preparation of intact proximal tubules in suspension, amiloride inhibits net Na^+ transport by direct inhibition of the activity of Na- and K-dependent adenosinetriphosphatase (Na,K–ATPase).

The tubules were prepared by collagenase digestion of the kidneys of female New Zealand White rabbits as reported (*9*), except that both kidneys (instead of a single kidney) were perfused in situ. The lumens of the tubules obtained by this procedure appear open by visual inspection (light microscopy) of fresh and fixed tissue. Amiloride inhibited the steady-state ouabain-sensitive rate of oxygen consumption (QO_2) in a dose-dependent manner (Fig. 1A). The effect of $10^{-3}M$ amiloride on the ouabain-sensitive QO_2 of tubules exposed to different concentrations of extracellular Na^+ is shown in Fig. 1B. We demonstrated earlier that this measurement is an accurate reflection of the Na pump activity due to the tight coupling between QO_2 and Na,K–ATPase activity (*10*). Furthermore, both QO_2 and Na,K–ATPase activity are directly related to the rate of transepithelial Na^+ transport (*11*). Thus, the amiloride-induced decrease in QO_2 reflects an inhibition of transepithelial Na^+ transport.

Transepithelial Na^+ transport may be described as a two-step process: passive Na^+ entry into the kidney cells across the apical membrane, followed by active Na^+ extrusion by the Na pump located at the basolateral membrane (*12*). Since the rate-limiting step of Na^+ transport in this preparation at physiological and subphysiological concentrations of extracellular Na^+ is the cellular entry of Na^+ across the apical membrane, the Na pump is limited with respect to the intracellular Na^+ concentration (*13*). Therefore, the function shown by the closed circles in Fig. 1B represents the response

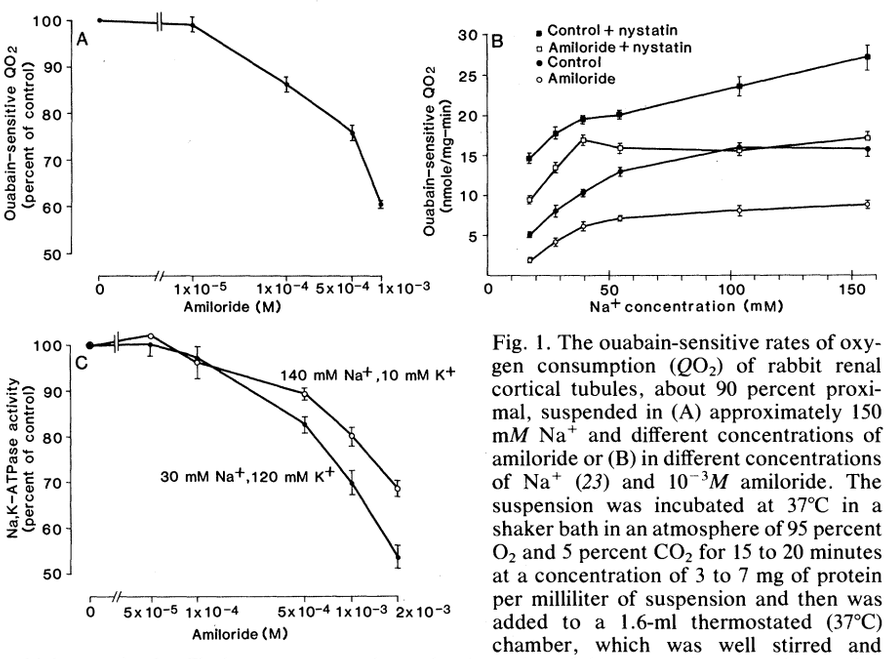

Fig. 1. The ouabain-sensitive rates of oxygen consumption (QO_2) of rabbit renal cortical tubules, about 90 percent proximal, suspended in (A) approximately 150 mM Na^+ and different concentrations of amiloride or (B) in different concentrations of Na^+ (*23*) and $10^{-3}M$ amiloride. The suspension was incubated at 37°C in a shaker bath in an atmosphere of 95 percent O_2 and 5 percent CO_2 for 15 to 20 minutes at a concentration of 3 to 7 mg of protein per milliliter of suspension and then was added to a 1.6-ml thermostated (37°C) chamber, which was well stirred and which contained a Clark-type oxygen electrode. A portion of the suspension was taken at this time to measure the extracellular and cellular Na^+ and K^+ (see Table 1). The tubules were exposed to amiloride just before the 37°C incubation in the shaker bath. First the QO_2 of control or amiloride-exposed tubules was measured, and then the QO_2 in the presence of nystatin (0.05 to 0.08 mg per milligram of protein) and ouabain ($10^{-4}M$), added at separate times to the same chamber, was measured. Points and vertical bars represent the means ± standard error (S.E.M.) of six to ten individual determinations. In (A), the control QO_2 is 17.5 ± 0.3 nmole of O_2 per milligram of protein per minute. (C) The response of the Na,K–ATPase activity of proximal tubule membranes to different concentrations of amiloride. ATPase activity was determined by the liberation of inorganic phosphate (P_i), which was extracted into an organic solution and measured spectrophotometrically (*24*). The ATPase assay was performed at 37°C and *p*H 7.4. The total ATPase activity was determined in the presence of 140 mM NaCl and 10 mM KCl or of 30 mM NaCl and 120 mM KCl, to which was added 5 mM ATP (Sigma, vanadate-free), 20 mM tris (Sigma), 5 mM $MgCl_2$, and 0.125 mM EGTA. The Mg-ATPase activity was determined by incubating the membranes in the above mixture plus $10^{-3}M$ ouabain (Sigma). The Na,K–ATPase activity was defined as the difference between the total and the Mg-ATPase activities. Samples were assayed in triplicate, and appropriate blanks were used to measure the nonenzymatic hydrolysis of P_i. The points and vertical bars represent the mean ± S.E.M. of three samples for all conditions, except one where no bars are shown (N = 2). Control Na,K–ATPase activities in nanomoles of P_i per milligram of protein per minute were 84.8 ± 7.1 for 30 mM Na^+ and 333.8 ± 10.9 for 140 mM Na^+.

of the Na pump to various concentrations of intracellular Na^+ that are produced by the different concentrations of extracellular Na^+. Amiloride inhibited the ouabain-sensitive QO_2 by about 50 percent (open circles in Fig. 1B). Several mechanisms can account for this inhibitory action, including inhibition of Na^+ entry, inhibition of cellular metabolism (for example ATP synthesis), and direct inhibition of the Na,K–ATPase activity. Additional experiments were performed to investigate these possiblilities.

Nystatin, a polyene antibiotic that increases the permeability of sterol-containing membranes to monovalent cations (*14*), can be used to enhance the entry of Na^+ and thus to raise the intracellular Na^+ concentration to nearly that of extracellular Na^+. When added to the suspension of proximal tubules, nystatin stimulates the steady-state QO_2 by causing an increase in the intracellular Na^+ concentration (*10*). Under these conditions, ouabain-sensitive nystatin-stimulated QO_2 (closed squares in Fig. 1B) reflects the Na pump activity in the presence of different presumed intracellular Na^+ concentrations. These rates are inhibited by $10^{-3}M$ amiloride (open squares in Fig. 1B). Since the cellular entry of Na^+ is not rate-limiting under these conditions, the inhibition in the presence of nystatin indicates that the action of amiloride is not due to an inhibition of Na^+ entry.

We also measured the effect of $10^{-3}M$ amiloride on the Na^+ and K^+ contents of tubules in suspension in the absence of nystatin (*15*). The Na^+ and K^+ contents of tubules exposed to amiloride were larger and smaller, respectively, than the corresponding contents of tubules not exposed to amiloride (Table 1). If the primary action of amiloride is to inhibit Na^+ entry (possibly by inhibition of Na^+-H^+ exchange), the expected result would be decreased Na^+, as was found in various epithelial tissues (*16*). Thus, both the inhibition of QO_2 in the presence of nystatin and the observed alterations in tissue ion content indicate that the primary action of amiloride in the intact tubule was to produce an inhibition of the activity of the Na pump.

Such an inhibition could be the result of a direct effect on the Na,K–ATPase enzyme or to an indirect effect, such as metabolic limitation. Therefore, we examined the effect of the drug on the Na,K–ATPase activity of membranes from our proximal tubule preparation (*17*). Amiloride inhibited the Na,K–ATPase activity in a dose-dependent manner (Fig. 1C). The inhibition by

Table 1. The tissue ion content of proximal tubules exposed to 157 mM Na^+ in the presence and absence of $10^{-3}M$ amiloride. Measurements were made as described (*17*). Each value represents the mean ± S.E.M. ($N = 4$). The effect of amiloride was highly significant by paired t-test.

Item	Tissue ion content (nmole/mg)	
	Na^+	K^+
Control	188.8 ± 63.7	313.1 ± 9.1
Amiloride	245.1 ± 68.0	253.8 ± 15.9
Paired Δ	+56.3 ± 18.4*	−59.3 ± 10.4†

*$P < .05$. †$P < .01$.

$10^{-3}M$ amiloride, the dose used in the oxygen consumption studies, was 20 percent in the presence of 140 mM Na^+ and 10 mM K^+ and 30 percent in the presence of 30 mM Na^+ and 120 mM K^+, the effect being significantly greater in the presence of the lower Na^+ concentration. The mean inhibition of QO_2 (27.3 ± 3.9 percent, $N = 6$; the mean at each Na^+ concentration was weighted equally) is in general agreement with the inhibition of Na,K–ATPase activity obtained at this dose (Fig. 1C). In the presence of amiloride, the ouabain-insensitive QO_2 of the tubules and the Mg-ATPase activity of the membrane fragments were not significantly different from the rates in the absence of amiloride. Thus, amiloride appears to inhibit pump activity by a direct inhibition of Na,K–ATPase activity. In these experiments, with tubules in suspension, amiloride was in contact with both the luminal and basolateral membranes. Since amiloride penetrates biological membranes (*18*), we cannot directly ascertain the sidedness of its action.

Under control conditions the Na pump operates at about 50 percent of its maximal rate (*19*), its activity being limited by the intracellular Na^+ concentration. The present finding that amiloride causes diminished Na pump activity as well as an increased Na^+ content in the intact tubules demonstrates that the primary effect of amiloride is on the Na pump. Inhibition of the Na^+ entry pathway would have produced an inhibition of Na pump activity accompanied by a diminished Na^+ content. The increase in intracellular Na^+ produced by amiloride was not of sufficient magnitude to stimulate Na pump activity to its level in the absence of amiloride. Presumably, the higher Na^+ (and lower K^+) concentration caused a diminished gradient for Na^+ entry, leading to a new steady state in which Na^+ transport was inhibited despite higher cellular Na^+, although part of the inhibition of Na^+ entry could have been due to a secondary action of amiloride on Na^+-H^+ exchange.

Amiloride also inhibits Na,K–ATPase in the rat salivary duct epithelium (*20*), cat pancreas (*21*), and human cardiac membranes (*22*) and is an important tool for the investigation of membrane transport properties. In studies of proximal apical membrane vesicles with an imposed transmembrane Na^+ or H^+ gradient, amiloride appears to inhibit Na^+-H^+ exchange specifically (*7*). However, under physiological conditions, high concentrations of amiloride inhibit Na^+ transport by a direct inhibition of the Na,K–ATPase of the intact proximal tubule. Under these conditions, amiloride is not a specific inhibitor of Na^+-H^+ exchange.

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23. Solution A (in millimoles per liter): NaCl, 105; $NaHCO_3$, 25; NaH_2PO_4, 2; $CaCl_2$, 1; KCl, 5; Mg_2SO_4, 1; glucose, 5; sodium lactate, 4; sodium glutamate, 5; sodium malate, 5; alanine; 1, TMA (tetramethylammonium, Eastman Kodak Co.) butyrate, 1; dextran, 0.6 percent; *p*H 7.4. Solution B: choline chloride (Sigma), 105; choline bicarbonate (Sigma), 25; H_3PO_4, 2; $CaCl_2$, 1; KCl, 5; Mg SO_4, 1; glucose, 5; TMA lactate, 4; TMA glutamate, 5; TMA malate, 5; alanine, 1; TMA butyrate, 1; dextran, 0.6 percent; *p*H 7.4. The Na^+ concentration was varied by mixing solution A and solution B in various proportions.
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H-2–Linked Resistance to Mastocytoma in Male Mice: Immune Response to a Histocompatibility Antigen on the X Chromosome

Abstract. *Male hybrids from a cross between female mice of strain C57BL/6Kh and males of strain DBA/2J lived longer after injection of P815 mastocytoma cells of DBA/2 origin than did their female siblings. Responses to the histocompatibility antigen on the X chromosome of the DBA/2 strain may be involved in resistance to the tumor. When the female parent was replaced with a C57BL/6Kh carrying one of several mutations in the* H-2 *region, this sex effect disappeared in some of the hybrid combinations. Thus, the* H-2 *complex appears to be involved in the regulation of the immune response to the X-linked histocompatibility antigen in this tumor model.*

In the mouse, the histocompatibility gene on the X chromosome (*H-X*), unlike that on the Y chromosome (*H-Y*) (*1*), has remained relatively obscure. Since the discovery by Bailey (*2*) of an X chromosome–determined skin graft incompatibility between two inbred mouse strains, C57BL/6 and BALB/c, few reports have appeared about the *H-X* locus (*3–8*). The *H-X* histoincompatibility between C57BL/6 and BALB/c was confirmed in skin graft experiments between reciprocal F_1 hybrids (*3*) and has also been shown between the following pairs of strains: A and C57BL/6 (*3–7*), A and BALB/c (*8*), DBA/2 and BALB/c (*8*), and DBA/2 and C57BL/10 (*8*). The *H-X* locus of the mouse is more polymorphic than the *H-Y* locus (*9*) and appears to have at least four alleles.

In studies of the genetic control of the resistance to the DBA/2 mastocytoma P815-X2 in histocompatible F_1 mice (hybrid resistance), we generally used recipients bearing the *H-X* allele of DBA/2 ($H\text{-}X^d$) so that there was no *H-X* incompatibility between tumor and recipients; indeed, no sex differences in resistance were detected (*10*). Recently, however, we observed that male (C57BL/6Kh × DBA/2J)F_1 hybrids (B6D2F_1) ($H\text{-}X^b/H\text{-}Y$) survived significantly longer than their female siblings ($H\text{-}X^b/H\text{-}X^d$) after injection of 10^6 viable P815-X2 tumor cells (*11*), suggesting that responses to $H\text{-}X^d$ may be involved in the resistance of these hybrids to the tumor cells.

The theoretical role for X-linked histoincompatibility in the male B6D2F_1 hybrids is illustrated in Fig. 1. Data reported here (Table 1) led us to the conclusion that we were detecting an anti-$H\text{-}X^d$ response to the tumor. Statistical comparisons between survival of male and female B6D2F_1 hybrids after injection of the tumor cells (*12*) revealed a significant difference in resistance to the tumor, with males surviving longer than females (experiment 1 in Table 1 and Fig. 2). In some cases this anti-$H\text{-}X^d$ response may have resulted in rejection of the tumor, since all survivors of the experiments were males (see footnotes to Table 1). Consistent with our hypothesis was the observation that

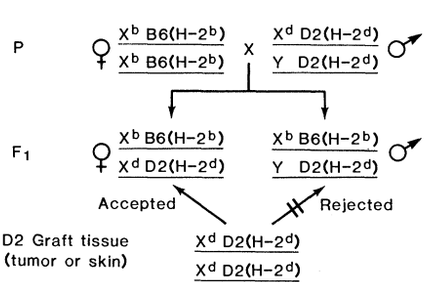

Fig. 1. The theoretical role for incompatibility against $H\text{-}X^d$ in male B6D2F_1 hybrids. The autosomal genome (including *H-2*) is represented as a single chromosome pair.

this sex effect was absent in homozygous DBA/2J controls and in (DBA/2J × C57BL/6Kh)F_1 hybrids (D2B6F_1) (experiment 3 in Table 1). Differences in median survival times between identical strains in separate experiments may reflect numerous variables and therefore cannot be legitimately compared. We report that strains C57BL/6 (B6) and DBA/2 (D2) may be *H-X*–incompatible, as has already been shown for C57BL/10 (closely related to B6) and D2 (*8*).

Hybrid resistance in mice is linked to the *H-2* complex on chromosome 17 (*13*). The introduction of B6 *H-2* mutants with various point mutations of single genes of the $H\text{-}2^b$ haplotype ($B6^m$) provided an opportunity to examine the effects of single genes on tumor resistance. By comparing the survival of $B6^mD2F_1$ females with B6D2F_1 females after injection of P815-X2 cells, we found that differences in a single gene (for example, *H-2K*) could profoundly influence survival, either positively or negatively (see footnotes to Table 1). Since several of the mutant gene products differ from the wild type by only one or a few amino acids (*14*), these small changes in H-2 antigens apparently can alter resistance to a histocompatible tumor, producing an effect similar to that of an immune response gene (*15–17*).

Analysis of the survival of male and female mutant hybrids showed that the anti-$H\text{-}X^d$ phenomenon is not present in all strains. Hybrids with *bm6, bm10, bm11, bm12, bm13*, and *bm16* mutations demonstrated a statistically significant sex effect ($P < .05$; Mantel-Cox logrank test). The male hybrids always survived longer than the females. Hybrids with *bm1, bm4, bm7, bm8*, and *bm14* mutations did not exhibit a statistically significant sex effect (experiments 1 and 2 in Table 1). This apparent variability in the immune response to $H\text{-}X^d$ among mutant hybrids, differing only in *H-2* and with theoretically identical X incompatibilities, suggests that responses to $H\text{-}X^d$ in this system are influenced by the *H-2* genotype of the recipient.

In bm7 and bm8 hybrids the anti-$H\text{-}X^d$ response, if present, may have been simply masked by the strong responses to the tumor (anti-P815-X2 response), as demonstrated by their increased survival over B6D2F_1 controls (footnotes to Table 1). This, however, cannot explain the reduction and perhaps the absence of the sex effect in *bm1, bm4*, and *bm14* hybrids, since they did not exhibit significant differences in survival in comparison with B6D2F_1 controls (*15–17*). Fur-