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EFFECT OF ALDOSTERONE ANTAGONISTS ON ALDOSTERONE-INDUCED ACTIVATION OF Mg^{2+}–HCO_3^- – ATPase AND CARBONIC ANHYDRASE IN RAT INTESTINAL MUCOSA

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Summary—In previous studies, Mg^{2+}-dependent, HCO_3^--activated ATPase in the brush border and carbonic anhydrase in the cytoplasm of rat duodenal and jejunal mucosa decreased after adrenalectomy. Both enzyme activities increased to near normal levels 4 h after i.p. injection of aldosterone (40 μg/kg). These results suggest the possibility that both enzymes in the small intestinal mucosa may be mediators of the action of aldosterone. In the present studies, therefore, the effects of actinomycin D (500 μg/kg, i.p.), spironolactone (50 mg/kg, s.c.) and potassium canrenoate (50 mg/kg, s.c.) on aldosterone-induced activation of both enzymes in the upper small intestinal mucosa from adrenalectomized rats were examined to clarify the mechanism of action of aldosterone in enzyme levels. Actinomycin D inhibited carbonic anhydrase activity in small intestinal mucosa from normal rats 4 h after i.p. injection but had no effect on ATPase activity, while two other drugs had no effect on either enzyme activity in normal rats up to 4 h later. Pretreatment with these 3 drugs 1 h before aldosterone administration (40 μg/kg, i.p.) to adrenalectomized rats blocked the aldosterone-induced activation of ATPase and carbonic anhydrase in the upper small intenstine. On the other hand, adrenalectomy and administration of aldosterone and its antagonists, alone or in combination, had no effect on kidney enzyme activities. These results confirm that Mg^{2+}–HCO_3^- – ATPase and carbonic anhydrase are mediators of the action of aldosterone in the upper small intestinal mucosa.

INTRODUCTION

Aldosterone is a potent regulator of transcellular sodium transport in many epithelia including mammalian nephron, toad bladder and amphibian skin [1, 2]. Rat intestinal mucosa, especially duodenal mucosa, has also been assumed to be a target organ of aldosterone because of the presence of [^{3}H]aldosterone binding receptors [3–5]. Other investigators have observed also that aldosterone promotes the absorption of Na^+ and secretion of K^+ through intestinal mucosa *in vivo* [6–8]. Watts and Wheldrake[9, 10] reported that the RNA and protein synthesis in rat intestinal mucosa decreased after adrenalectomy and that aldosterone administration restored them to normal levels. These observations seem to suggest that aldosterone acts on intestinal mucosa through the induction of DNA-dependent RNA synthesis followed by the synthesis of some enzyme proteins that mediate the ion transport through the mucosal membrane. However, the direct examination of the effect of aldosterone and its antagonists on extra-renal organs, especially on intestinal mucosal enzymes, has not been reported clearly to date.

Mg^{2+}–HCO_3^- – ATPase and carbonic anhydrase in rat intestinal mucosa are assumed to be related to the membrane transport of H^+, Cl^-, and HCO_3^- [11, 12]. We reported recently that Mg^{2+}–HCO_3^- – ATPase in microvilli of rat duodenal mucosa was found to have the highest specific activity among all organs [13]. Furthermore, both ATPase and carbonic anhydrase activities in the upper small intestinal mucosa decreased after adrenalectomy and replacement with aldosterone, but not with dexamethasone, restored the decreased enzyme activities to near normal levels [14]. These results seem to indicate that these two enzymes have an interrelationship with aldosterone and also exert an effect on intestinal function.

It is well known that actinomycin D antagonizes aldosterone-induced acceleration of sodium transport in many epithelia through inhibition of protein synthesis [15, 16]. Spironolactone is also an aldosterone antagonist through competition for its receptor sites [17]. Rossier *et al.*[18] have shown the antagonistic effect of spironolactone on aldosterone-induced increase in RNA synthesis and Na^+ transport in toad bladder. Spironolactone also competitively inhibited the aldosterone binding to kidney mineralocorticoid receptors [19–21]. Potassium canrenoate is a water-soluble spironolactone analogue and increases the urinary Na/K excretion ratio in aldosterone-treated adrenalectomized rats and also antagonizes [^{3}H]aldosterone binding to its receptor sites *in vitro*, though its potency is lower than that of spironolactone [22]. In spite of these studies, however, the relationship between aldosterone and aldosterone

antagonists for intestinal enzyme levels has not been clarified.

In the present series of experiments, the effects of actinomycin D, spironolactone and potassium canrenoate on Mg^{2+}–HCO_3^- – ATPase and carbonic anhydrase activities of several organs, especially duodenal mucosa, from normal, adrenalectomized and aldosterone-treated adrenalectomized rats were examined to clarify the interaction between aldosterone and these drugs at the enzyme level.

EXPERIMENTAL

Animals

Laboratory-bred male Wistar rats (300–350 g) were used. Bilateral adrenalectomy was performed under ether anaesthesia 5 days prior to experiment. Adrenalectomized rats were maintained on a commercial solid diet (Oriental Co. Japan) and 0.9% saline *ad libitum* at room temperature (23 ± 2°C). Normal rats were maintained on the same diet and tap water *ad libitum* under the same conditions.

Chemicals

Disodium adenosine-5′-triphosphate (Na_2ATP; Boehringer Mannheim) was dissolved in distilled water and adjusted to pH 8 with Tris-base. D-Aldosterone (Merck) was dissolved in 99.5% ethanol and diluted with saline to a concentration of 40 μg/ml. The final concentration of ethanol was about 1.0%. Normal and adrenalectomized control rats were injected i.p. with 0.9% saline containing an equal concentration of ethanol. Actinomycin D (Cosmegen; Merck-Banyu Co. Japan) was dissolved in saline to a concentration of 500 μg/ml and injected i.p. Spironolactone (Sigma) was first dissolved in 99.5% ethanol, diluted with an equal volume of saline and injected s.c. The final concentration of spironolactone injected was 10 mg/ml. Normal and adrenalectomized control rats were injected s.c. with the same amount of 50% ethanol. Potassium canrenoate (Soldactone; Searle Co.) was dissolved in saline to a concentration of 20 mg/ml and injected s.c. Other chemicals used for the preparation of enzyme samples, and for assay of its enzyme activity, were all reagent grade.

Sample preparation procedures

Animals were fasted for about 20 h with access to drinking fluids before sacrifice. Under pentobarbital anaesthesia (Nembutal sodium; Abbott Lab., North Chicago, 30 mg/kg, i.p.), the abdomen was opened and the entire intestine was perfused via the portal vein and abdominal aorta with cold heparin–saline. The duodenal and upper jejunal portions, each approx 12 13 cm in length, were excised from the pylorus. The colon and rectum was combined and used as a large intestinal sample. The right kidney was removed, decapsulated and pieces of cortex were used as a kidney sample. The intestinal lumen from each rat was flushed with cold saline, and the mucosa from each segment was scraped off with a glass slide and weighed. Tissues from 2 rats were pooled to get a single sample.

The crude brush border fraction was prepared by a calcium precipitation procedure similar to that described by Schmitz *et al.*[23] and Malathi *et al.*[24]. In brief, samples were homogenized in 10 ml ice-cold 50 mM D-mannitol–2 mM Tris–HCl buffer (pH 7.1 at 4°C). Homogenates were filtered through a piece of nylon mesh and aliquots of this homogenate were taken for the determination of enzyme activity and protein content. Thereafter, 1 M $CaCl_2$ solution was added to the residual homogenate to a final concentration of 10 mM and the mixture was gently stirred in ice-water for 10 min. The homogenates were then centrifuged at 3,000 *g* for 15 min in a refrigerated centrifuge. The supernatant was carefully decanted and centrifuged at 43,000 *g* for 20 min. The resultant supernatant was decanted and used as a carbonic anhydrase sample. Pellets were rehomogenized in the same mannitol–Tris buffer and were recentrifuged at 43,000 *g* for 20 min. The supernatant was discarded and pellets (crude brush border containing fraction) were homogenized with the same buffer and used as Mg^{2+}–HCO_3^- – ATPase sample. All samples were stored at −20°C until use.

Enzyme assay

Mg^{2+} and Mg^{2+}–HCO_3^- – ATPase activity in homogenate and brush border was assayed in a manner similar to that described previously [14]. Aliquots of sample were placed in test-tubes (15 × 100 mm) with 1.0 mM $MgCl_2$, 50 mM $NaHCO_3$, 3 mM ATP and 50 mM Tris–HCl buffer (pH 9.0 at 37°C) in a total volume of 1.0 ml. After incubation at 37°C for 10 min in a shaking water bath, test-tubes were placed in ice-water and the reaction was stopped by adding 1 ml 20% trichloroacetic acid. The precipitates were removed by centrifugation and the inorganic phosphate (Pi) liberated was determined by the method of Allen [25] with a slight modification described by Nakamura[26]. Enzyme activity was expressed as μmol Pi/mg protein/h. Mg^{2+}–ATPase activity was assayed without $NaHCO_3$.

Carbonic anhydrase activity in the homogenate and supernatant fraction was assayed by the modified colorimetric method [27] of Wilbur and Anderson[28]. Enzyme activity was calculated according to the following expression, where enzyme activity = $(T_0 - T)/T$/mg protein, where T_0 is the reaction time for the uncatalyzed reaction and T represents the time intervals for the catalyzed reaction.

Protein assay

Protein in enzyme sample was determined by the method of Lowry *et al.*[29] with bovine serum albumin as standard.

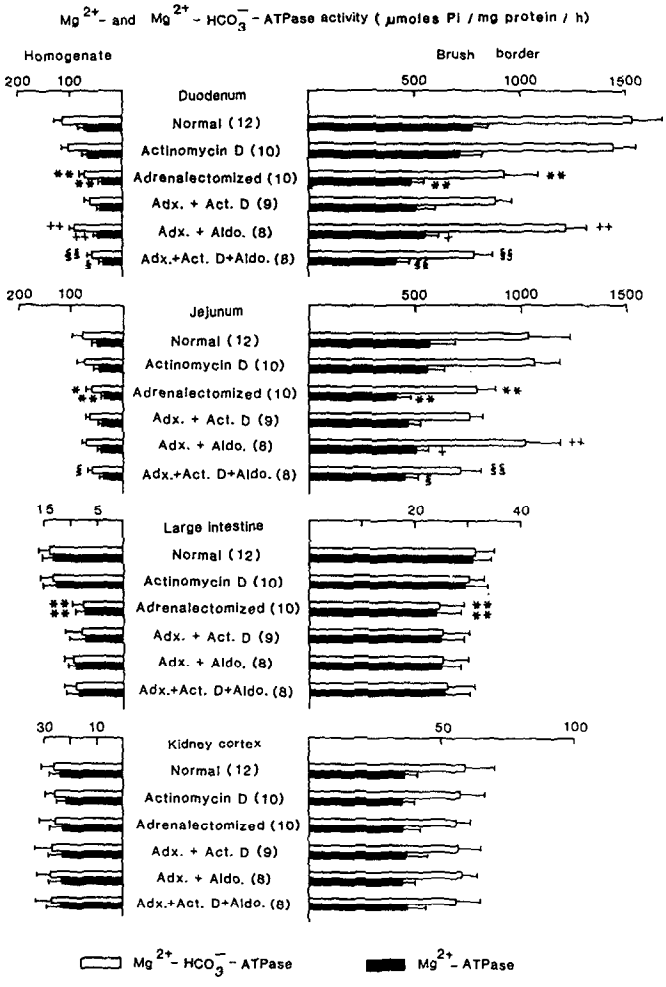

Fig. 1. Effect of actinomycin D (500 μg/kg, i.p.) and aldosterone (40 μg/kg, i.p.), administered separately or concurrently, on Mg^{2+} and Mg^{2+}–HCO_3^- – ATPase activities in duodenal, jejunal, large intestinal mucosa and kidney cortex. The number of samples is shown in parentheses. Each column represents the mean value ± SD. *,**Significantly different from normal group (*$P < 0.05$, **$P < 0.01$). †,††Significantly different from adx. group (†$P < 0.05$, ††$P < 0.01$). §§§Significantly different from aldosterone treated group (§$P < 0.05$, §§$P < 0.01$).

Statistical analysis

Student's t-test was used to test for statistical significance.

RESULTS

Effect of actinomycin D

Figure 1 shows the effect of actinomycin D on Mg^{2+} and Mg^{2+}–HCO_3^- – ATPase activity in homogenate and 43,000 g precipitate (crude brush border) from normal, adrenalectomized, and aldosterone-treated adrenalectomized rats. In normal and adrenalectomized rats, actinomycin D in a dose of 500 μg/kg had no significant effect on the enzyme activity in any organ 4 h after i.p. injection. Aldosterone in a dose of 40 μg/kg increased the small intestinal ATPase activity of adrenalectomized rats towards (duodenum) or to normal (jejunum) levels 4 h after i.p. administration. Pretreatment with actinomycin D 1 h prior to aldosterone administration inhibited the aldosterone-induced increases in enzyme activity in duodenum and jejunum, but had no effect in large intestine and kidney.

Figure 2 shows the changes in carbonic anhydrase activity in homogenate and 43,000 g supernatant under the same experimental conditions indicated in Fig. 1. In normal rats, a single injection of actinomycin D in a dose of 500 μg/kg, i.p., significantly decreased carbonic anhydrase activity in the duodenum and jejunum with no effect in the large intestine or kidney. Adrenalectomy decreased enzyme activity in duodenum, jejunum and large intestine, but there was no alteration in the kidney. Replacement of aldosterone in a dose of 40 μg/kg, i.p., after 4 h, restored the decreased enzyme activity to near normal levels. Furthermore, pretreatment with actinomycin D 1 h prior to aldosterone administration

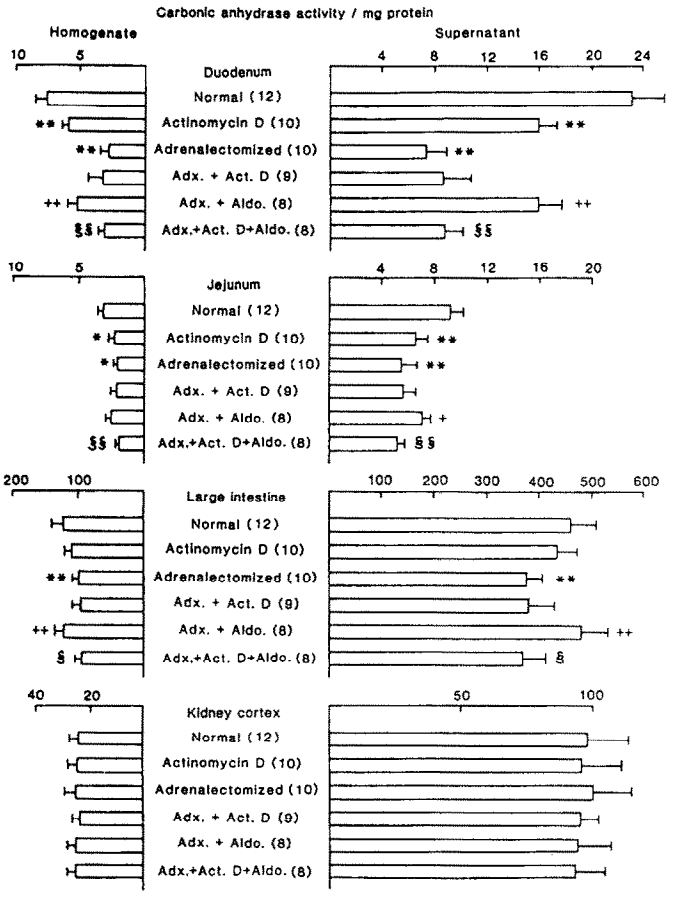

Fig. 2. Effect of actinomycin D (500 μg/kg, i.p.) and aldosterone (40 μg/kg, i.p.), administered separately or concurrently, on carbonic anhydrase activity in duodenal, jejunal, large intestinal mucosa and kidney cortex. The number of samples is shown in parentheses. Each column represents the mean value $\pm$ SD. *,**Significantly different from normal group ($*P < 0.05$, $**P < 0.01$). †,††Significantly different from adx. group ($\dagger P < 0.05$, $\dagger\dagger P < 0.01$). §§Significantly different from aldosterone treated group ($\S P < 0.05$, $\S\S P < 0.01$).

inhibited the aldosterone-induced activation of carbonic anhydrase in the duodenum, jejunum and large intestine with no effect in the kidney.

Effect of spironolactone

Figure 3 shows the effect of spironolactone on Mg^{2+}-HCO_3^- – ATPase activity from normal, adrenalectomized, and adrenalectomized and aldosterone treated rats. A single injection of spironolactone in a dose of 50 mg/kg, s.c., had no effect on ATPase activity in normal or adrenalectomized rats 4 h after injection. However, pretreatment with the same dose of spironolactone 1 h prior to aldosterone administration inhibited the aldosterone-induced activation of Mg^{2+} and Mg^{2+}-HCO_3^- – ATPase in duodenal and jejunal mucosa, but had no effect in large intestinal mucosa and kidney. Figure 4 shows the changes in carbonic anhydrase activity after the administration of spironolactone, alone or in combination with aldosterone. In normal and adrenalectomized rats, a single injection of spironolactone in a dose of 50 mg/kg, s.c., had no effect on carbonic anhydrase activity in any organ. However, pretreatment with this drug 1 h prior to aldosterone administration inhibited the aldosterone-induced activation of the enzyme in duodenum, jejunum and large intestine.

At autopsy, bleeding and inflammation was observed at the injection sites of spironolactone solution or its solvent alone, however, these injuries had no effect on ATPase and carbonic anhydrase activities.

Effect of potassium canrenoate

Figure 5 indicates the effect of potassium canrenoate on Mg^{2+} and Mg^{2+}-HCO_3^- – ATPase activity from normal, adrenalectomized, and adrenalectomized and aldosterone treated rats. A single injection of potassium canrenoate into normal and adrenalectomized rats (50 mg/kg, s.c.) had no effect on ATPase activity up to 4 h later. However, pretreatment with this drug 1 h before aldosterone administration blocked the aldosterone-induced activation of ATPase in duodenum and jejunum with no

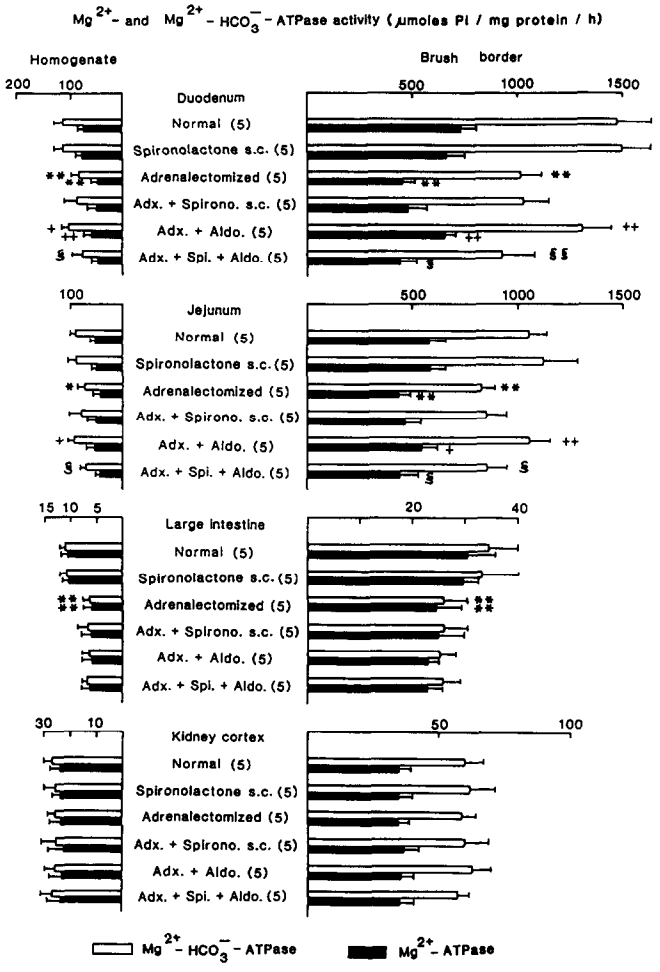

Fig. 3. Effect of spironolactone (50 mg/kg, s.c.) and aldosterone (40 μg/kg, i.p.), administered separately or concurrently, on Mg^{2+} and Mg^{2+}–HCO_3^- – ATPase activities in duodenal, jejunal, large intestinal mucosa and kidney cortex. The number of samples is shown in parentheses. Each column represents the mean value ± SD. *,**Significantly different from normal group (*$P < 0.05$, **$P < 0.01$). †,††Significantly different from adx. group (†$P < 0.05$, ††$P < 0.01$). §,§§Significantly different from aldosterone treated group (†$P < 0.05$, §§$P < 0.01$).

effect in large intestine and kidney. Figure 6 shows the changes in carbonic anhydrase activity under the same experimental conditions represented in Fig. 5. Although potassium canrenoate itself had no effect on carbonic anhydrase activity from normal and adrenalectomized rats, pretreatment with this drug 1 h before aldosterone administration blocked the stimulating effect of aldosterone on duodenal enzyme activity. However, the antagonistic effect of potassium canrenoate on aldosterone action was slightly less than that of spironolactone.

DISCUSSION

According to Feldman *et al.*[1] and Fanestil and Kipnowski[30], the possible action of aldosterone can be considered under the following four categories: (1) induction of RNA synthesis; (2) induction of mitochondrial enzymes coupled with the generation of ATP; (3) induction of permeases which may mediate the apical Na^+ permeability; (4) induction of the basolateral membrane enzyme, Na^+–K^+–ATPase. The "permease hypothesis" introduced by Crabbé[31] and Sharp and Leaf[32] suggests that the binding of aldosterone to a physiological receptor in the target tissue induces extensively protein synthesis, and that the newly synthesized protein acts as a permease and facilitates the entry of Na^+ into the tissue through the mucosal membrane. Most studies performed to examine such a mechanism of action have employed mammalian kidney and toad bladder. Thus the proteins synthesized in response to aldosterone have not been extensively studied. These proteins may be involved in the mediation of ion transport through intestinal mucosal membrane; the number of experiments using intestinal mucosa is,

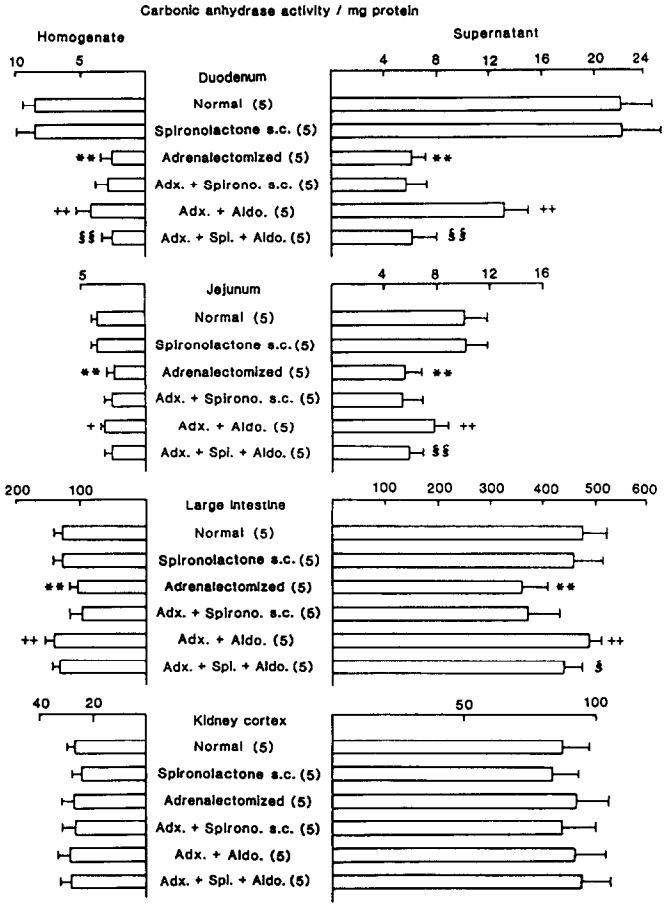

Fig. 4. Effect of spironolactone (50 mg/kg, s.c.) and aldosterone (40 μg/kg, i.p.), administered separately or concurrently, on carbonic anhydrase activity in duodenal, jejunal, large intestinal mucosa and kidney cortex. The number of samples is shown in parentheses. Each column represents the mean value $\pm$ SD. **Significantly different from normal group ($P < 0.01$). †††Significantly different from adx. group ($\dagger P < 0.05$, $\dagger\dagger P < 0.01$). §§§Significantly different from aldosterone treated group ($\S P < 0.05$, $\S\S P < 0.01$).

however, small compared with studies on mammalian kidney and toad bladder.

In the present series of experiments, we have confirmed that the Mg^{2+}–HCO_3^- – ATPase and carbonic anhydrase in the duodenal and jejunal mucosa are sensitive to adrenalectomy and aldosterone replacement. The decreases in carbonic anhydrase and Mg^{2+}–HCO_3^- – ATPase activity from normal values 5 days after adrenalectomy were about 60–70% for the former and 30–40% for the latter. The decrease in carbonic anhydrase activity in duodenal mucosa following adrenalectomy is greater than any other known enzyme examined to date. Furthermore, aldosterone in doses of 5 μg, 20 μg and 40 μg/kg, i.p., after 4 h, significantly increased these decreased carbonic anhydrase activities in dose-dependent fashion [33, 14]. Aldosterone in doses of 5 μg and 20 μg/kg had a tendency to increase Mg^{2+}–HCO_3^- – ATPase activity [33] and 40 μg/kg of aldosterone significantly increased this ATPase activity in upper small intestinal mucosa of adrenalectomized rats [14]. In contrast, dexamethasone in doses of 0.2 mg, 1 mg and 5 mg/kg, i.p., after 4 h, had no replacement effect on ATPase and carbonic anhydrase activities in upper small intestinal mucosa of adrenalectomized rats [33, 14]. Recently we confirmed also that corticosterone in doses of 1 mg and 4 mg/kg had no significant effect on Mg^{2+}–HCO_3^- – ATPase and carbonic anhydrase activities of duodenal, jejunal, large intestinal mucosa and kidney cortex in adrenalectomized rats within 4 h after i.p. injection (unpublished). These results, therefore, seem to suggest the importance of mineralocorticoids, e.g. aldosterone, in maintaining both enzyme activities.

In the previous paper [34], the authors offered the hypothesis that Mg^{2+}–HCO_3^- – ATPase and carbonic anhydrase in duodenal mucosa may regulate the membrane transport of Cl^-, HCO_3^-, Na^+, and H^+ through Cl^-–HCO_3^- and Na^+–H^+ exchange mechanisms. In the present study, these enzyme activities decreased following adrenalectomy, and administration of aldosterone restored them to near

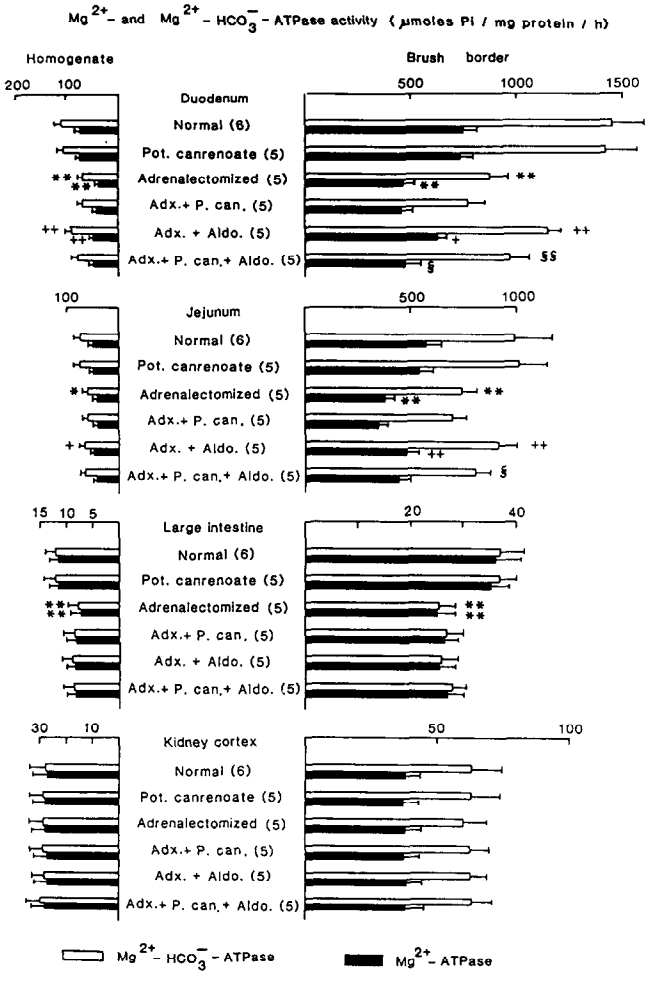

Fig. 5. Effect of potassium canrenoate (50 mg/kg, s.c.) and aldosterone (40 μg/kg, i.p.), administered separately or concurrently, on Mg^{2+} and Mg^{2+}–HCO_3^- – ATPase activities in duodenal, jejunal, large intestinal mucosa and kidney cortex. The number of samples is shown in parentheses. Each column represents the mean value ± SD.
*,**Significantly different from normal group (*$P < 0.05$, **$P < 0.01$). †,††Significantly different from adx. group (†$P < 0.05$, ††$P < 0.01$). §,§§Significantly different from aldosterone treated group (§$P < 0.05$, §§$P < 0.01$).

normal levels. In general, aldosterone is thought mainly to be related to the membrane transport of cations, e.g. Na^+, K^+, and H^+. However, the results obtained in this study seem to indicate that the aldosterone also may regulate the anion transport through intestinal mucosal membrane. To answer these questions, more precise experiments on the metabolism or movement of not only cations but also anions through intestinal mucosal membrane after adrenalectomy and aldosterone replacement may be necessary in the future.

Actinomycin D was first reported as an inhibitor of the antinatriuretic effect of aldosterone in adrenalectomized rats [35] and now is known as an inhibitor of aldosterone-induced RNA and protein synthesis. In the present series of experiments, actinomycin D completely blocked the activation of Mg^{2+}–HCO_3^- – ATPase and carbonic anhydrase by aldosterone. Furthermore, we have observed previously [14] that cycloheximide, a protein synthesis inhibitor, inhibited Mg^{2+}–HCO_3^- – ATPase and carbonic anhydrase activities in duodenal mucosa of normal rats and also blocked the activating effect of aldosterone on duodenal enzymes from adrenalectomized rats. In the present study, actinomycin D in a dose of 500 μg/kg inhibited carbonic anhydrase activity in duodenal and jejunal mucosa of normal rats. In contrast, the same dose of actinomycin D had no effect on Mg^{2+}–HCO_3^- – ATPase activity in normal rats. Actinomycin D in a dose of 1 mg/kg also did not show any effect on Mg^{2+}–HCO_3^- – ATPase activity of duodenal mucosa in normal rats (unpublished). If Mg^{2+}–HCO_3^- – ATPase is one of the target enzymes of aldosterone, a similar change to that in carbonic anhydrase may be expected according to the "induction hypothesis" of aldosterone

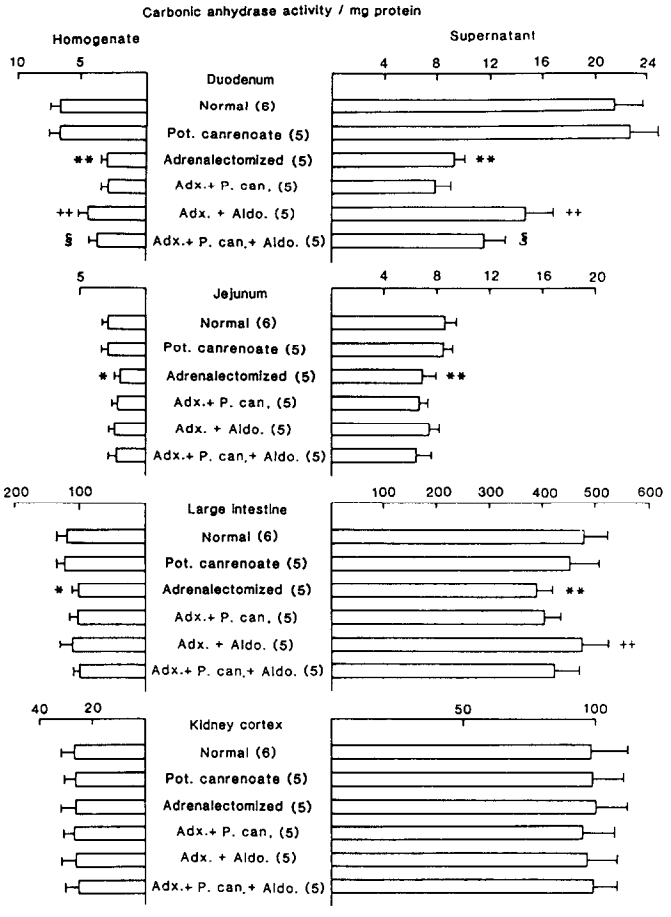

Fig. 6. Effect of potassium canrenoate (50 mg/kg, s.c.) and aldosterone (40 μg/kg, i.p.), administered separately or concurrently, on carbonic anhydrase activity in duodenal, jejunal, large intestinal mucosa and kidney cortex. The number of samples is shown in parentheses. Each column represents the mean value $\pm$ SD.
*,**Significantly different from normal group (*$P < 0.05$, **$P < 0.01$). ††Significantly different from adx. group ($P < 0.01$). §Significantly different from aldosterone treated group ($P < 0.05$).

action. The reason of these inconsistent phenomena is not clear at present and must be clarified in the future.

Spironolactone acts at the first step in the so-called "induction" by aldosterone and inhibits subsequent steps including RNA and protein synthesis [20, 21]. Recently, Geheb *et al.*[36] reported that the *in vitro* concentration of spironolactone that abolished aldosterone-stimulated Na^+ transport also inhibited aldosterone-induced protein synthesis. In the present series of experiments, the antagonism by spironolactone of aldosterone-induced activation of Mg^{2+}–HCO_3^- – ATPase and carbonic anhydrase in the upper small intestinal mucosa was demonstrated in Figs 3 and 4. However, spironolactone itself in a dose of 50 mg/kg, s.c., had no effect on Mg^{2+}–HCO_3^- – ATPase or carbonic anhydrase activity in normal rats. The oral administration of spironolactone (20 mg/kg) to normal rats once daily for 4 days also had no effect on enzyme activity (data not shown). According to the studies by Geheb *et al.*[36], Campen *et al.*[37] and Elmslie *et al.*[38], several hundred times as much spironolactone as the dose of aldosterone is required to inhibit aldosterone-stimulated Na^+ transport. It is thought that in the present series of experiments, the dose of spironolactone administered to normal rats may be relatively small compared with the concentration of endogeneous aldosterone and therefore does not appear to have an inhibitory effect on enzyme activity.

Potassium canrenoate is a water-soluble spironolactone analogue and is metabolized into canrenone [39, 40]. Neurath and Ambrosius[41] have reported canrenone to be one of the major metabolites of spironolactone; Merkus *et al.*[42], in contrast, reported canrenone to be only a minor metabolite of spironolactone. Although potassium canrenoate also blocked aldosterone-induced activation of ATPase and carbonic anhydrase in duodenal and jejunal mucosa in the present series of experiments, its inhibiting effect seems to be slightly less than that of spironolactone. Casals-Stenzel *et al.*[43] reported that

the antialdosterone potency of potassium canrenoate on the urinary Na/K ratio of adrenalectomized rats was less than that of spironolactone. On the other hand, potassium canrenoate, a water-soluble aldosterone antagonist, may be more useful in laboratory experiments than spironolactone which is water-insoluble.

The noteworthy difference between Mg^{2+}–HCO_3^- – ATPase and carbonic anhydrase in the large intestine was that the decrease levels of carbonic anhydrase following adrenalectomy were restored to normal by aldosterone replacement, but Mg^{2+}–HCO_3^- – ATPase activity was not affected by aldosterone replacement.

Since kidney is a main target organ of aldosterone in mammals, some effects of aldosterone-antagonists were expected, but no changes in enzyme activity were observed following adrenalectomy and the administration of aldosterone, actinomycin D, spironolactone and potassium canrenoate, alone or in combination.

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