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1,2-Dimethylhydrazine-induced alterations in Na^+–H^+ exchange in rat colonic brush-border membrane vesicles

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1,2-Dimethylhydrazine, in weekly subcutaneous (s.c.) doses of 20 mg/kg body weight, produces colonic tumors in virtually 100% of rodents, with a latency period of approximately 6 months. To determine whether alterations in Na^+–H^+ exchange existed before the development of dimethylhydrazine-induced colon cancer, rats were given s.c. injections of this agent (20 mg/kg body wt. per week) or diluent for 5 weeks. Animals were then killed, rat colonic brush-border membrane vesicles prepared and amiloride-sensitive sodium-stimulated proton efflux was measured and compared in control and treated-preparations. The results of these studies demonstrated that dimethylhydrazine treatment: (1) significantly increased the V_{max} of this exchange without altering the K_m for sodium of this exchange process, utilizing the fluorescent pH-sensitive dye, acridine orange; ^{22}Na flux experiments also demonstrated an increase in amiloride-sensitive proton-stimulated sodium influx across treated-membrane vesicles; (2) did not appear to significantly influence Na^+ permeability or proton conductance in treated-preparations compared to their control counterparts; and (3) did not significantly affect the kinetic parameters of amiloride-sensitive sodium-stimulated proton efflux in renal cortex brush-border membrane vesicles using acridine orange. This data, therefore, suggests that alterations in Na^+–H^+ exchange in rat colonic brush-border membranes may be involved in the malignant transformation process induced by this procarcinogen in the large intestine.

Prior studies by our laboratory, utilizing the 1,2-dimethylhydrazine model of colonic adenocarcinoma, have shown that alterations in the lipid composition, lipid fluidity and phospholipid methyltransferase activity could be detected in rat colonic brush-border membranes after 5–15 weeks administration of this procarcinogen, i.e., before the development of colon cancer [1].

In this regard, in the past few years the role of ionic exchange between tumor cells and their environment has received considerable interest [2]. Various cations, especially protons, have been reported to be involved in the mitogenic response elicited by growth factors [2]. Particular attention has been focused on the involvement of Na^+–H^+ exchange in the malignant transformation process [2,3]. This ubiquitous carrier-mediated transport system couples the movement of Na^+ into the cell with extrusion of protons out of the cell resulting in cellular alkalinization. Several investigators [2,4,5] have proposed that this alkalinization is critical to the induction of cellular responses following mitogenic stimulation in normal and neoplastic cells.

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Recently, our laboratory [6–9] and others [10] have demonstrated the existence of an amiloride-sensitive Na^+–H^+ exchanger (antiporter) in rat colonic brush-border membrane vesicles, using both ^{22}Na and pH-sensitive fluorescent dye techniques. This antiporter possesses properties similar to Na^+–H^+ exchangers previously described in other plasma membranes [6–10]. Moreover, this transport system's activity appears to be modulated by alterations in the lipid composition, lipid fluidity and transmethylation activity of rat colonic brush-border membrane vesicles [7–9]. In view of our previous findings of dimethylhydrazine-induced 'premalignant' alterations in these membrane parameters, it was of interest to determine whether changes in the Na^+–H^+ exchanger could be detected in apical membrane vesicles of rats administered this agent for 5 weeks. To further establish a relationship between changes in this colonic exchanger and malignant transformation, since dimethylhydrazine has not been shown to cause kidney tumors [11], it was also of interest to determine whether this agent induced alterations in Na^+–H^+ exchangers in renal cortex brush-border membrane vesicles. The results described below demonstrate that the colonic but not renal antiporter's activity is altered by treatment with this procarcinogen and serves as the basis for the present report.

Albino male rats of the Sherman strain weighing 75–100 g were given weekly s.c. injections of diluent or 1,2-dimethylhydrazine dihydrochloride (Sigma Chemical Co., St. Louis, MO) at a dose of 20 mg/kg body wt. for 5 weeks as described [1]. Control and dimethylhydrazine-treated animals were fasted for 18 h with water ad libitum before they were killed. For each control or dimethylhydrazine-treated colonic membrane preparation, eight animals were killed rapidly by cervical dislocation, their colons excised and the cecum from each animal discarded. Epithelial cells were then obtained [12] and used to isolate brush-border membranes as previously described [12]. The purity of each colonic membrane preparation was assessed by the marker enzymes alkaline phosphatase (*p*-nitrophenylphosphatase) and cysteine-sensitive alkaline phosphatase [12]; specific activity ratios (original homogenates) ranged from 14 to 17 for these enzymes in each preparation and did not significantly differ between control and treated-preparations. The corresponding values for succinate dehydrogenase, NADPH–cytochrome-*c* reductase, and sodium-potassium-dependent adenosine triphosphatase, marker enzymes for mitochondrial, microsomal and basolateral membranes, respectively, ranged from 0.50 to 1.40 in each of these preparations and did not differ significantly between control and treated-membranes.

In certain experiments, renal cortical brush-border membrane vesicle preparations were prepared from control and treated-animals, as described by Harris et al. [13]. The purity of each of these renal membrane preparations was assessed by the marker enzyme alkaline phosphatase [13]; specific activity ratios ranged from 15 to 18 for this enzyme in each preparations and did not significantly differ between control and treated-membranes. In agreement with previous studies [13], these membranes were found to be minimally contaminated by basolateral membranes or intracellular membranes as assessed by appropriate marker enzymes (see above). Protein was estimated by the method of Lowry et al. [14], using bovine serum albumin as standard.

Amiloride-sensitive sodium-stimulated proton efflux was measured in rat colonic and renal cortex brush-border membrane vesicles, using the pH-sensitive dye, acridine orange, as previously described by our laboratory [6–9] and others [15–17]. A Perkin-Elmer 650-40 spectrofluorometer (Perkin Elmer Corp., Norwalk, CT) (excitation 493 nm, emission 530 nm) equipped with a thermostated cuvette, stirring system and adding port was used in these studies. All experiments were performed at 26 °C.

The assay solution contained 6 μM acridine orange, 250 mM sucrose, 100 mM *N*-methyl-glucamine gluconate and 10 mM Tris-Hepes (pH 7.5). After 2 ml of this buffer reached steady-state fluorescence (approx. 90 s), 50 μl of brush-border membrane vesicles (100–150 μg protein), preloaded with 250 mM sucrose, 100 mM *N*-methyl-glucamine gluconate and 10 mM Tris-Hepes (pH 6.0) were added. As previously noted [17,18], there was a 25–35% quenching in acridine orange fluorescence signal which reached equilibrium within 2 min. Sufficient amounts of sodium gluconate

solution were then added to achieve a final concentration of 2.5–50 mM in the assay medium, which resulted in a collapse of the outwardly directed H^+ gradient and a reappearance of acridine orange fluorescence [15]. The increase in fluorescence was linear for at least 2 s and the initial rate of acridine orange fluorescence reappearance was measured as the initial slope. After 300 s the pH gradient was dissipated with 150 mM potassium gluconate and 10 μg nigericin as described [19]. The small fluorescence quenching still remaining after nigericin addition was due to binding of the dye to the membranes [16]. Appropriate corrections were made for this binding as described [20].

Kinetics of the Na^+–H^+ exchange process were evaluated in control and dimethylhydrazine-treated rat colonic brush-border membrane vesicle preparations by determining the effect of increasing sodium concentrations (2.5–50 mM) on sodium-stimulated proton efflux with acridine orange fluorescence techniques. The exchange process demonstrated saturation kinetics for control and treated-preparations, and at each concentration of sodium tested was higher for treated-preparations and was inhibited by amiloride (1 mM) approx. 85% (not shown). As shown in Fig. 1, where substrate versus rate were plotted in double-reciprocal form [21], the results were linear and revealed a similar K_m for sodium in control (17.6 ± 2.5) and treated (21.9 ± 4.4) preparations ($N = 3$). In contrast, the values for V_{max}, expressed in arbitrary fluorescence units, were found to be significantly higher for treated-preparations (2541 ± 219, $N = 3$) than their control counterparts (1971 ± 83, $N = 3$) ($P < 0.05$).

Amiloride-sensitive Na^+–H^+ exchange in rat colonic membrane vesicles was also studied in ^{22}Na influx experiments. Uptake of ^{22}Na was measured at 26 °C by a Millipore filtration technique as described by Murer et al. [22]. The incubation medium contained 144 mM KCl, 5 mM Mes, 13 mM Hepes, 13 mM Tris, 1 mM NaCl (pH 7.5) ±1 mM amiloride. The experiment was started by the addition of 160 μl of the incubation media containing 1–2 μCi of ^{22}Na +40 μl of the membrane suspension (pH 6.0 or pH 7.5 inside). After designated periods of time (1 min and 180 min), the reaction was terminated by the addition

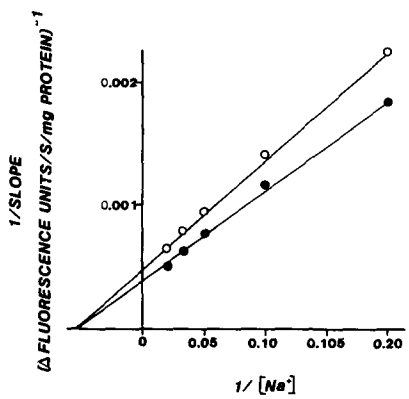

Fig. 1. Amiloride-sensitive sodium-stimulated proton efflux was measured in rat colonic brush-border membrane vesicles, using the pH-sensitive fluorescent dye, acridine orange, as described under Methods and Materials. Representative double-reciprocal plots of three separate experiments, using membrane vesicles prepared from the colon of control (○) and dimethylhydrazine-treated (●) animals, are shown. See text for further details.

of 5 ml of ice-cold stop solution containing 150 mM LiCl, 10 mM Hepes, 10 mM Tris (pH 7.5). The diluted sample was immediately filtered through a 0.45 μm Millipore filter (HAWP), and the filter was washed three times with 5 ml of cold stopping solution. Filters were dissolved in scintillation fluid, and the radioactivity measured in a Beckman LS-6800 scintillation counter [22]. Each experiment was performed in triplicate on three separate preparations.

In agreement with the acridine orange studies cited above, amiloride-sensitive, pH-stimulated sodium influx using ^{22}Na at 1 min was significantly higher in the dimethylhydrazine-treated colonic brush-border membrane vesicles in the presence of a pH gradient (pH 7.5 outside, pH 6.0 inside) (0.91 ± 0.07 nmol/mg protein, $N = 3$) than in control vesicles (0.57 ± 0.05, $N = 3$) ($P < 0.01$). Accummulation of Na^+ after 180 min of incubation, at a time when both Na^+ and H^+ gradients were dissipated, however, was similar in the vesicles of both groups (control 0.43 ± 0.05; dimethylhydrazine-treated 0.40 ± 0.06 nmol/mg protein, $N = 3$) ($P > 0.05$). As previously discussed [23], the latter finding indicates that the average intravesicular volumes of vesicles isolated from control and treated-animals were compara-

ble, suggesting that the differences in the rates of uptake seen using acridine orange and ^{22}Na techniques (see above) were not due to alterations in vesicle size. Since similar aliquots of membrane protein were used in the transport experiments, this also rules out changes in vesicular membrane number as being responsible for the Na^+–H^+ exchange differences seen in the present studies between control and treated-preparations.

Moreover, when these ^{22}Na influx studies were performed in rat colonic membrane vesicles using 1 mM NaCl in the absence of a pH gradient (pH 7.5 inside and outside), no differences were noted between control (0.19 ± 0.04, $N = 3$) and treated-vesicles (0.22 ± 0.03 nmol/mg protein, $N = 3$) ($P > 0.05$), indicating that dimethylhydrazine treatment did not appear to influence Na^+ permeability in these preparations [23].

In order to assess the possible effect of dimethylhydrazine on proton conductance, the formation of a pH gradient by an outwardly directed K^+ gradient and valinomycin was measured as previously described using acridine orange [24]. Briefly, membrane vesicles preloaded with a buffer containing 100 mM potassium gluconate (pH 7.5) were added to an incubation medium containing 100 mM *N*-methylglucamine gluconate (pH 7.5) in the presence and absence of valinomycin (10 μg per mg protein). Generation of the pH gradient was followed by measuring the magnitude of the quench of acridine orange fluorescence [24]. No differences were noted in the generation of pH gradient in the presence of valinomycin in control and treated-vesicles (data not shown), indicating proton conductance was also not influenced by administration of this procarcinogen.

Kinetics of the Na^+–H^+ exchange process were also evaluated in control and dimethylhydrazine-treated rat renal cortex brush-border membrane vesicle preparations by determining the effect of increasing sodium concentration (2.5–5.0 mM) on sodium-stimulated proton efflux with acridine orange techniques. As with colonic membrane vesicles (see above), the exchange process demonstrated saturation kinetics for kidney control and treated-membrane preparations. In contrast to our findings in the colonic vesicles, however, no differences were noted in the exchange process in control and treated-vesicles at each sodium concentration tested (not shown). When substrate versus rate were plotted in double-reciprocal form [21], the results were linear and revealed a similar K_m for sodium in control (5.5 ± 1.1) and treated (5.0 ± 0.9) preparations ($N = 3$) as well as similar values for V_{max}, expressed in arbitrary fluorescence units (control 1166 ± 82; treated 1197 ± 58) ($N = 3$).

As noted above, previous studies from our laboratory have shown that: (1) administration of dimethylhydrazine for 5 weeks produced an increase in rat colonic brush-border membrane fluidity [1]; and (2) alterations in the fluidity of rat colonic plasma membranes were associated with changes in Na^+–H^+ exchange activity [7–9]. It was, therefore, of interest to examine the fluidity of treated and control colonic membrane preparations in the present studies using steady-state fluorescence polarization techniques and the lipid-soluble fluorophore DL-12-(9-anthroyl)stearic acid (12-AS) as previously described by our laboratory [1]. In agreement with earlier studies [1], the *r* values of 12-AS of treated-preparations (0.066 ± 0.001) were significantly lower than control values (0.072 ± 0.001, $N = 3$) ($P < 0.05$), indicating that treated-vesicles were more fluid than their control counterparts.

It should be noted that the dimethylhydrazine treatment regimen used in the present experiments (20 mg/kg body wt. per week) produces colonic tumors, but not kidney tumors, in virtually 100% of rodents, with a latency period of approximately 6 months [11,25]. The alterations in the activity of Na^+–H^+ exchange noted in these studies in the colonic treated-preparations were seen after only 5 weeks of dimethylhydrazine administration, i.e., before the development of colonic adenocarcinomas. Whether these dimethylhydrazine-induced alterations in the Na^+–H^+ exchanger are truly related to the malignant transformation process seen in this organ will require additional studies. In this regard, however, the finding that the changes in Na^+–H^+ exchange induced by dimethylhydrazine were seen in the brush-border membranes of the colon and not brush-border membranes of the kidney cortex do suggest that these alterations may, indeed, be related to the malignant transformation process in the colon. Recent studies in other laboratories [2,26] also

lend support to this contention. Davies et al. [26] have recently shown that amiloride-sensitive sodium transport is increased in the distal colon of CF_1 mice administered dimethylhydrazine for 4 weeks. Furthermore, treatment with this agent had no effect on distal colonic amiloride-sensitive sodium transport in DBA/2 mice, a strain which does not develop colonic malignant transformation. Lagarde and Pouyssegur [2] have also demonstrated that the tumor incidence of several cultured tumor cell Na^+–H^+ antiportless mutants, implanted into Balb/c athymic nude mice, was reduced by 80–100%. This data indicates that the loss of a functional Na^+–H^+ antiporter was detrimental to the development of these tumor cells and again suggests that this exchange process may be intimately involved in malignant transformation.

At this time, the mechanism(s) responsible for the increased activity of the Na^+–H^+ exchanger in colonic brush-border membrane vesicles prepared from rats treated with dimethylhydrazine are also unclear. Based on the present findings, it does not appear that dimethylhydrazine influenced the activity of this exchanger by altering the proton/sodium permeability of treated preparations. Since Na^+/K^+-ATPase activity was similar in both control and treated-preparations it is also unlikely that this agent produced a loss of plasma membrane polarity in colonic cells which, in turn, led to a redistribution of the carrier system. In this regard, in agreement with earlier studies by our laboratory [1], the present studies have shown that the fluidity of treated-colonic brush-border membrane vesicles was greater than their control counterparts after 5 weeks of dimethylhydrazine administration. This is particularly interesting in view of earlier studies [7–9] which have shown that a direct correlation appears to exist between rat colonic apical membrane vesicular fluidity and Na^+–H^+ exchange. It would, therefore, seem reasonable to suggest that alterations in fluidity of these membrane might be responsible for the present dimethylhydrazine-induced changes in the Na^+–H^+ antiporter activity.

Other possible mechanisms for this phenomenon, however, will also need to be studied further to clarify this issue. For example, dimethylhydrazine might have a direct effect on the antiporter. Alternatively, this procarcinogen might alter Na^+–H^+ exchange in these apical membranes by influencing the sensitivity of a possible non-transporting H^+ site. While the latter has not yet been shown to exist in rat colonic apical membranes, such a site has recently been shown to be present in the Na^+–H^+ exchanger of renal brush-border membrane vesicles [27]. It is, therefore, possible that dimethylhydrazine, like several other stimuli of Na^+–H^+ exchange in various cell types [28], might induce an alkaline shift of the pH_i-dependence curve of approx. 0.2 to 0.3 units and, thereby, activate this exchanger. Along these same lines, Doppler et al. [3] have previously shown that Ehrlich ascites tumor cells can regulate their internal pH by altering the affinity of the Na^+–H^+ antiporter for internal protons. Further experiments will, therefore, be necessary to elucidate the exact mechanism(s) whereby dimethylhydrazine influences this exchanger. Regardless of the mechanism(s) involved, however, it does appear that dimethylhydrazine can influence Na^+–H^+ exchange in rat colonic brush-border membrane vesicles.

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