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The effects of cycloheximide on Na^+/H^+ antiporter activity in cultured opossum kidney cells

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These studies examined the effects of cycloheximide on the Na^+/H^+ antiporter in cultured opossum kidney cells. The effects of cycloheximide on antiporter activity depended on the basal level of activity. These data suggest that the Na^+/H^+ antiporter may be regulated by several processes which are sensitive to protein synthesis inhibition.

The transport rate of the Na^+/H^+ antiporter and Na^+/phosphate cotransporter can be acutely decreased by parathyroid hormone and 8-Br-cAMP in renal proximal tubule cells [1] and in an established cell line from opossum kidney (OK) [2–5]. Long term regulation of transport could also be achieved at the level of synthesis of the entire transporter, or of critical regulatory subunits. The present studies characterized the effects of protein synthesis inhibition with cycloheximide on activity of the Na^+/H^+ antiporter in OK cells. We found that the activity of the antiporter was inhibited by 8-Br-cAMP, and that this effect was not modified by inhibition of protein synthesis. When OK cells were treated with cycloheximide for 5 h, the changes in Na^+/H^+ antiporter activity were inversely related to the basal level of transport.

Abbreviations: 8-Br-cAMP, 8-bromo adenosine 3′,5′-cyclic monophosphate; DMSO, dimethyl sulfoxide; OK cells, opossum kidney cells.

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OK cells were provided by Dr. J. Handler of the NIH, and were grown to confluence in Costar 24 well clusters in 95% air, 5% CO_2, modified Eagles' minimal essential medium with Earle's salts + 2 mM glutamine and 10% fetal calf serum. Cells in passage 76–90 were used in the present experiments. Cycloheximide in dimethyl sulfoxide (DMSO) was added to the experimental wells in a final concentration of 70 μg/ml, 5 h before each uptake experiment. Four hours before the uptake studies, the medium was replaced with a sodium-free Hepes buffered medium containing 150 mM *N*-methyl-D-glucamine chloride, 5.6 mM potassium chloride, 1.2 mM calcium chloride, 1.0 mM magnesium chloride, 20 mM Hepes, and 10 mg/ml sodium-free dialyzed bovine serum albumin (fraction V), adjusted to pH 7.5 at 37°C, with or without cycloheximide (70 μg/ml), and with or without 0.1 mM 8-Br-cAMP.

10 min prior to uptake studies, the cells were acid loaded by the potassium-nigericin method [6] in 147 mM *N*-methyl-D-glucamine chloride, 3.0 mM potassium chloride, 1.2 mM calcium chloride, 1.0 mM magnesium chloride, 20 mM Hepes, 1 mM phosphate, 1 mM ouabain and nigericin (3 μg/ml) adjusted to pH 7.5 at 37°C. ^{22}Na uptake

studies were then performed for 2 min at an extracellular sodium concentration of 10 mM with 5 μCi/ml ^{22}Na in medium containing 140 mM *N*-methyl-D-glucamine chloride, 5.6 mM potassium chloride, 1.2 mM calcium chloride, 1.0 mM magnesium chloride, 20 mM Hepes, 1.0 mM phosphate, 2.0 mM amiloride (where indicated), and 1.0 mM ouabain, adjusted to pH 7.5 at 37° C. The medium was always iso-osmotic (adjusted with *N*-methyl-D-glucamine chloride) so that changes in cell volume were avoided. Na^+/H^+ antiporter activity was defined as the difference between total ^{22}Na uptake, and that in the presence of 2.0 mM amiloride. The uptake reactions were terminated by washing the monolayers with iced 100 mM magnesium chloride plus 0.1 mM amiloride. The cell monolayers were then dissolved in 0.1% SDS. Samples were counted in a scintillation counter and analyzed for protein content. Each data point was done in triplicate, and the results are reported as nmoles uptake/min per mg protein.

The first experiments examined the effects of 8-Br-cAMP and cycloheximide on Na^+/H^+ antiporter activity in OK cells. There was a $47.6 \pm 3.7\%$ decrease in Na^+/K^+ antiporter activity when OK cells were treated with 0.1 mM 8-Br-cAMP (from 17.0 ± 2.3 nmol/min per mg protein to 9.2 ± 1.8 nmol/min per mg protein; $P < 0.001$, $n = 9$ paired experiments). The inhibitory effects of 8-Br-cAMP on Na^+/H^+ antiporter and Na^+/phosphate cotransport have been previously reported in OK cells. Similar results have also been obtained with parathyroid hormone, forskolin, and phosphodiesterase inhibitors [2–4], demonstrating that both of these sodium-coupled transporters are regulated by cAMP in OK cells. In contrast to the uniform effects of 8-Br-cAMP, cycloheximide did not appear to affect the Na^+/H^+ antiporter (from 17.0 ± 2.3 nmol/min per mg protein to 20.1 ± 1.8 mmol/min per mg protein; $n = 9$ paired experiments). Further studies revealed that cycloheximide did not prevent the inhibitory effect of 8-Br-cAMP. When added to OK cells already exposed to cycloheximide, 8-Br-cAMP inhibited Na^+/H^+ antiporter activity by $38.9 \pm 6.7\%$ (to 12.1 ± 1.6 nmol/min per mg protein), similar to the inhibition seen with 8-Br-cAMP in cells which had not been exposed to cycloheximide (9.2 ± 1.8 nmol/min per mg protein). (Toback and coworkers reported that incubation with 50 μg/ml cycloheximide for 1 h inhibited incorporation of [^{3}H]leucine into acid-insoluble material by 85% in a monkey kidney cell line [7].) The lack of effect of cycloheximide on the 8-Br-cAMP response suggests that activation of cAMP-dependent protein kinase inhibits both transporters in the OK cells, and that this effect does not require ongoing protein synthesis. Caverzasio et al. [3] also found that cycloheximide did not diminish the inhibitory effect of parathyroid hormone on Na^+/phosphate cotransport in OK cells.

The Na^+/phosphate cotransport system is enhanced in OK cells when phosphate is removed from the culture media [8,9], similar to the effects of substrate deprivation on sodium-dependent amino acid transport system in fibroblasts [10,11]. Both the A system for amino acid transport and the Na^+/phosphate cotransporter are sodium-dependent, can be stimulated by substrate deprivation, and are inhibited by cycloheximide [8–11].

The amount of a protein in a cell is determined by its relative rates of synthesis and degradation. Complete inhibition of protein synthesis by cycloheximide should result in a decrease in the amount of a cellular protein proportionate to its rate of degradation. This reasoning suggests that the Na^+/H^+ antiporter is degraded very slowly because its activity was not inhibited by cycloheximide. However, the effects of cycloheximide on the Na^+/H^+ antiporter in OK cells may not be interpretable in terms of synthesis or degradation of a single protein. Identical treatment of different groups of cells on different days resulted in markedly altered levels of Na^+/H^+ exchange, and either stimulation or inhibition of the Na^+/H^+ antiporter by cycloheximide (Fig. 1). The dashed regression line in Fig. 1 depicts the relationship between the basal activity of the Na^+/H^+ antiporter and the response to cycloheximide. Cells with low control levels of antiporter activity (3–10 nmol/min per mg protein) showed stimulation (up to 80%) while cells with a high control levels of antiporter activity (20–40 nmol/min per mg protein) showed inhibition of Na^+/H^+ antiporter activity with cycloheximide.

This observation suggests that the Na^+/H^+ antiporter may be tonically inhibited by a protein with a more rapid rate of degradation than the

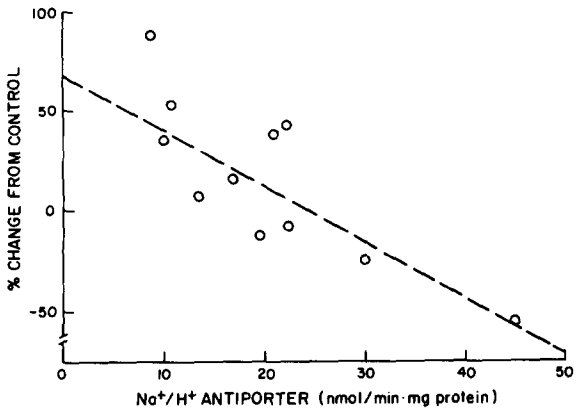

Fig. 1. Cycloheximide effect on Na^+/H^+ antiporter activity. The percent change in amiloride-sensitive sodium uptake in cycloheximide-treated cells compared to their paired controls is plotted as a function of basal amiloride-sensitive sodium uptake. The line represents the linear-regression analysis ($Y = -2.8\ (\pm 0.8)X + 67.8\ (\pm 26.6)$; $r = 0.746$).

antiporter itself. When present at high levels, this factor would result in a relatively low basal level of Na^+/H^+ antiporter activity (3–10 nmol/min per mg protein). Inhibition of protein synthesis with cycloheximide would decrease the activity of the regulatory factor while minimally affecting the number of transporters, leading to the observed increase in Na^+/H^+ antiporter activity. Conversely, when the activity of the regulatory factor is minimal, and a high basal level of Na^+/H^+ antiporter activity is observed, the effects of protein synthesis inhibition would be manifest on the antiporter itself.

The effects of cycloheximide on the Na^+/H^+ antiporter are similar to those on the insulin receptor described by Knutson et al. [12]; treatment with cycloheximide blocked inactivation of the insulin receptor, but did not affect its recycling or translocation. It was concluded that a protein factor with a rapid turnover was required for inactivation of the insulin receptor [12]. Thus, in OK cells, the Na^+/H^+ antiporter could be regulated by a similar factor, with stimulation of antiporter activity by cycloheximide reflecting a decrease in the activity of such an inhibitory factor.

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