Chronic Fluoride Ingestion Decreases ^{45}Ca Uptake by Rat Kidney Membranes[1]

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ABSTRACT High exposures to fluoride (F^-) may occur in environments rich in F^- from natural or industrial sources and from misuse of F^--containing dental care products, particularly by children. Both acute and chronic exposures to elevated levels of F^- have negative effects on several calcium-dependent processes, including kidney glomerular and tubular function. We examined the effect of chronic F^- ingestion on ATP-dependent ^{45}Ca uptake by rat kidney membrane vesicles to characterize the mechanism by which high F^- alters Ca^{++} transport in the kidney. Twenty weanling female Sprague-Dawley rats were raised on low-F^- (0.9 mg/L), semi-purified diet with a Ca^{++} concentration of 400 mg/100g diet. Rats were divided into four groups and were fed ad libitum deionized water containing F^- at 0, 10, 50, or 150 mg/L added as NaF for 6 wk. This consumption produced plasma F^- levels of <0.4, 2, 7, or 35 μmol/L, respectively. ATP-dependent ^{45}Ca uptake was significantly lower in the 150 mg F^-/L exposure group than in the 0 mg F^-/L controls ($P < 0.05$). Studies with thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{++}-pump, showed that the lower uptake was associated with significantly lower activities of both the plasma membrane Ca^{++}-pump ($P < 0.05$, 150 mg F^-/L group versus control) and endoplasmic reticulum Ca^{++}-pump ($P < 0.05$ for both the 50 and 150 mg F^-/L groups versus control). Slot blot analysis of kidney homogenates with specific Ca^{++}-pump antibodies showed less ($P < 0.05$) endoplasmic reticulum Ca^{++}-pump protein and plasma membrane Ca^{++}-pump protein in all treatment groups than controls. Both Ca^{++}-pumps are transport molecules of great importance in the regulation of Ca^{++} homeostasis. Our study suggests that chronic, high F^- ingestion producing high plasma F^- levels may occur in humans and may affect Ca^{++} homeostasis by increasing the turnover or breakdown or decreasing the expression of plasma membrane and endoplasmic reticulum Ca^{++}-pump proteins. J. Nutr. 129: 1209–1213, 1999.

KEY WORDS: • *Ca^{++}transport* • *fluoride* • *Ca^{++}-pump* • *endoplasmic reticulum Ca^{++}-pump* • *plasma membrane Ca^{++}-pump* • *rats*

With the exception of dental fluorosis, no association has been shown between exposure to low levels of fluoride (F^-)[3] in drinking water (1–2 mg/L) and adverse physical effects (Kaminsky et al. 1990). High exposure levels, however, are of concern. Skeletal fluorosis has been reported in populations exposed to 8–10 mg F^-/L for 10 y or more (Whitford 1992). Two epidemiological studies indicate that the incidence of bone fractures also correlates significantly with water fluoridation (Jacobsen et al. 1990, Sowers et al. 1991). The second of these two studies, by Sowers et al., showed a significant increase in fracture incidence for women 55–80 y of age. This increase occurred independent of the confounding variables of Ca^{++} intake, weight, size, age, and hormone usage.

High F^- exposures of 10 or more mg/L are attainable from environments rich in F^- from natural or industrial sources. High F^- exposure also occurs during therapeutic exposure to F^- for the treatment of osteoporosis (Pak et al. 1997). Of additional concern is the potential for high exposures, particularly of children, from the consumption of pleasant-tasting, F^--containing dental care products (Whitford 1989).

Das and Susheela (1993) studied the effects of long-term administration of F^- on intestinal absorption, urinary excretion, and plasma levels of Ca^{++} in rabbits and found clear systemic effects. Hypocalcemia was observed in F^- treated animals despite a drop in Ca^{++} excretion and an increase in intestinal Ca^{++} absorption. These findings suggest an increase in Ca^{++} retention by the tissues in chronic F^- toxicity. This increase in Ca^{++} retention would be consistent with the inhibition of a cellular Ca^{++} efflux mechanism as well. Andersen et al. (1986) produced a normocalcemic condition in F^- treated pigs fed a Ca^{++} and vitamin D-supplemented diet. This normocalcemic state, however, was also associated with a retention of Ca^{++} by tissues that was much higher than normal. In an effort to restore normocalcemia in osteofluorotic subjects by increasing Ca^{++}, the result may actually be the induction of osteosclerosis. Thus, osteomalacia and osteosclerosis may both be observed in osteofluorotic subjects as a function of dietary Ca^{++}.

Long-term exposure to elevated levels of F^- has negative effects on calcium-dependent processes in the kidney as well. For example, studies have shown varying degrees of tubular

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[3] Abbreviations used: DAB, diaminobenzidine tetrahydrochloride; F^-, fluoride; PMCA, plasma membrane Ca^{++}-pump.

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TABLE 1

Plasma F^- and Ca^{++} and body weight in rats consuming water containing various levels of fluoride for 6 wk[1]

Water F^-	*n*	Plasma F^-	Plasma Ca^{++}	Body wt
mg/L		*μmol/L*	*mmol/L*	*g*
0	5	0 ± 0.10	1.315 ± 0.074	179.2 ± 12.6
10	6	2 ± 0.73*	1.140 ± 0.045	175.0 ± 8.3
50	5	7 ± 1.17*	1.286 ± 0.024	179.6 ± 8.9
150	4	35 ± 23.45*	1.105 ± 0.050	163.4 ± 33.1

[1] Values are means ± SD
* Significantly different from 0 mg F^-/L water group.

dysfunction associated with high F^- exposure (Elsair et al. 1982, Herman 1956). Jolly et al. (1980) found a reduction in creatinine clearance in patients with chronic skeletal fluorosis, indicative of glomerular dysfunction.

In our study, we evaluated $^{45}Ca^{++}$ uptake by membrane vesicles prepared from the rat kidney cortex following 6 wk of chronic F^- ingestion from the drinking water. We selected exposure levels that would produce plasma concentrations of F^- attainable by humans from environmental exposures. After chronic exposure to F^-, rats were killed, and $^{45}Ca^{++}$ uptake was measured. To determine if chronic F^- exposure had an effect on Ca^{++} transport protein expression, independent of direct interaction between F^- and Ca^{++} transport protein activity, the uptake medium for these studies contained no added F^-.

MATERIALS AND METHODS

Chronic exposure of rats to F^-. Approval of the animal protocol was obtained from the Committee on Animal Use in Research and Education at the Medical College of Georgia. Twenty weanling female Sprague-Dawley rats (Harlan, Indianapolis, IN) were fed a semi-purified rat diet 76A, (Purina Test Diets, Richmond, IN) (American Institute of Nutrition 1993) for the duration of the study (6 wk). This diet was low in F^- (0.9 mg/L), as determined using the Model 9409 ion-specific electrode (Orion, Boston, MA) and contained 400 mg Ca^{++}/100 g diet, as measured on a Spectra AA atomic absorption spectrometer (Varian, Sunnyvale, CA). Rats were maintained in a controlled atmosphere environment at 23°C and 50% relative humidity. Rats were divided into four groups (see **Table 1** for *n* per group) and were fed ad libitum deionized water containing F^- at 0, 10, 50, or 150 mg/L added as NaF for 6 wk.

Analysis of plasma fluoride and calcium. Animals were anesthetized with ether prior to blood collection and killed via a pentobarbital overdose without regaining consciousness. Blood was collected from the right atrium of each rat and lightly heparinized. Plasma fluoride was analyzed using the ion-specific electrode (Orion, Model 9409) following overnight diffusion using the hexamethyldisiloxane-facilitated method of Taves (1968) as modified by Whitford (1996a). Reference standards for fluoride were prepared by us using the highest quality ACS-certified reagents available. Reference standards ranging from 0.25 nmol/L to 100 nmol/L were used for the fluoride determinations. Plasma ionic calcium was analyzed using the Microelectronics (Bedford, NH) Model MI-600 Mini-Calcium Electrode with Model MI-402 Micro-Reference Electrode, following the manufacturer's instructions. Reference standards for calcium determinations were prepared by us using the highest quality ACS-certified reagents available. Reference standards for calcium were prepared ranging from 10 μmol/L to 100 mmol/L.

^{45}Ca uptake into rat kidney mixed membrane vesicles. Following their removal, one kidney was frozen for slot blot analysis while the other kidney was used the same day to prepare mixed membrane vesicles without freezing. The cortex was cut from this kidney and homogenized with an Omni 1000 (Omni International, Waterbury, CT) for 1 min in 5 bursts of 12 s each in homogenization buffer containing 100 mmol KCl/L, 5 mmol $MgCl_2$/L, 50 mmolTris HCl/L (pH 7.2), 5 mmol benzamidine/L, 50 mg trypsin inhibitor/L, and 0.5 mmol dithiothreitol/L. Homogenates were centrifuged at 15,600 x *g* at 4°C for 10 min. The pellet was discarded, and the protein content of the supernatant was determined by the bicinchoninic acid method (Pierce, Rockford, IL). Volumes of supernatant containing 1.8 mg protein each were added to Airfuge™ tubes (Beckman Instruments, Palo Alto, CA), and the volume of each was adjusted to 200 μL. These tubes were centrifuged at 100,000 x *g* for 1 h at 4°C after which the supernatant was discarded. The mixed membrane pellets were then suspended in 150 μL uptake solution containing 130 mmol KCl/L, 34 mmol K-HEPES/L (pH 7.2), 2 mmol $MgCl_2$/L, 5 mmol K-oxalate/L, 100 μmol $CaCl_2$/L, and 110 μmol M EGTA/L, (free Ca^{++} = 1.6 μmol/L). To start the uptake reaction, 50 μL of this mixed membrane suspension was added to 500 μL of the same uptake solution, but at 37°C, in a shaker waterbath with 1.5 GBq/L $^{45}CaCl_2$ and, where appropriate, 5 mmol Tris ATP/L and 2 μmol thapsigargin/L. The uptake reaction was continued for 4 min and stopped by vacuum filtration of the membranes over 0.45 μm HAWP filters (Millipore, Bedford, MA). This time period was used because uptake by these vesicles proceeded in a linear fashion for 4 min. Filters were washed with 9 mL of wash solution containing 150 mmol KCl/L, 20 mmol K-HEPES/L (pH 7.4), and 2 mmol EGTA/L before placing them into scintillation vials for counting. Filters treated in the same way but without kidney membranes were also counted to calculate nonspecific background radioactivity.

Quantitation of plasma membrane and endoplasmic reticulum Ca^{++}-pumps by slot blot analysis. Quantitation of plasma membrane and endoplasmic reticulum Ca^{++}-pump epitopes in homogenates of rat kidney mixed membranes, involved immobilization of homogenate proteins on nitrocellulose via a Hybri-Slot slot blot apparatus (Bethesda Research Laboratories, Gaithersburg, MD). Samples of homogenate proteins (25 μg) in 50μL of homogenization buffer were loaded into each well of the slot blot apparatus with a vacuum and immobilized on the nitrocellulose. Wells were washed with 500 μL of 50 mmol Tris-HCl/L, pH 7.2. Antigen was visualized on nitrocellulose by the avidin-biotin-peroxidase technique.

Monoclonal antibody 5F10 was obtained from Dr. John Penniston of the Mayo Clinic, Rochester, MN. This antibody reacts with a highly conserved region of all plasma membrane Ca^{++}-pump (PMCA) isoforms. Monoclonal antibody IID8, specific for endoplasmic reticulum Ca^{++}-pump isoform SERCA2 was purchased from BIOMOL, (Plymouth Meeting, PA). Western blot analysis was performed (5F10, 1:1000 or IID8, 1:500) to confirm the specificity of each antibody. The same antibody dilutions were used for both Western blot and slot blot analysis. Diaminobenzidine tetrahydrochloride (DAB) was the substrate for the peroxidase molecule linked to the primary antibodies. The density of brown insoluble precipitate formed by the DAB was analyzed by scanning densitometry.

Data are expressed as means ± SE and were analyzed for significant differences using ANOVA. Where variances were unequal, natural log transformation of the data was employed. An α value of 0.05 was selected a priori. Multiple comparisons versus the control group were assessed by Dunnett's method.

RESULTS

The ingestion of deionized water containing F^- at 0, 10, 50, or 150 mg/L added as NaF for 6 wk produced plasma F^- concentrations ranging from <0.4 to 35 μmol/L (Table 1). Body weights and plasma ionic Ca^{++} concentrations did not differ among the groups.

ATP-independent Ca^{++} uptake and adsorption did not differ among groups **(Fig. 1)**. Similarly, in the presence of thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{++}-pump, no significant differences were observed (Fig. 1). ATP-dependent $^{45}Ca^{++}$ uptake without thapsigargin, however, was lower in vesicles prepared from rats with plasma F^- levels of 35 μmol/L than in the control group ($P < 0.05$).

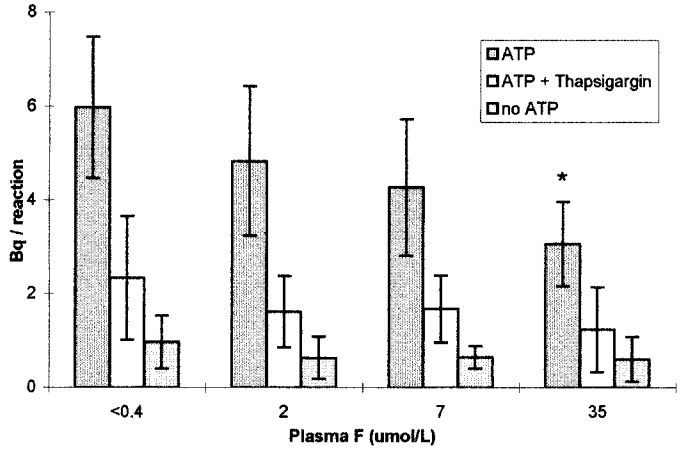

FIGURE 1 ATP-independent Ca^{++} uptake and adsorption (no ATP), ATP-dependent Ca^{++} uptake (ATP), and ATP-dependent Ca^{++} uptake in the presence of thapsigargin, a specific inhibitor of the endoplasmic reticulum Ca^{++}-pump (ATP + Thapsigargin), by kidney membrane vesicles from rats with plasma fluoride levels of <0.4, 2, 7, and 35 μmol F^-/L. Plasma fluoride concentrations were at these levels after 6 wk of consuming water containing 0, 10, 50, and 150 mg F^-/L, respectively. Values are means ± SD, n = 4–6 (see Table 1). *Significantly different ($P < 0.05$) from control (<0.4 μmol F^-/L).

Plasma membrane Ca^{++}-pump—dependent uptake was considered separately by subtraction of thapsigargin-sensitive and ATP-independent uptake from total uptake. The plasma membrane Ca^{++}-pump activity in the 35 μmol/L plasma F^- group was significantly lower than that of the <0.4 μmol/L plasma F^- level control group **(Fig. 2)**. Thapsigargin-sensitive uptake, representing endoplasmic reticulum Ca^{++}-pump, also was considered separately. When the <0.4 μmol/L plasma F^- control group was compared to the 7 μmol/L and 35 μmol/L plasma F^- groups, the lower Ca^{++} uptake in the high plasma F^- groups was associated with significantly lower activity of the endoplasmic reticulum Ca^{++}-pump.

The plasma membrane Ca^{++}-pump protein levels in all rats exposed to chronic high F^- were lower than the control group

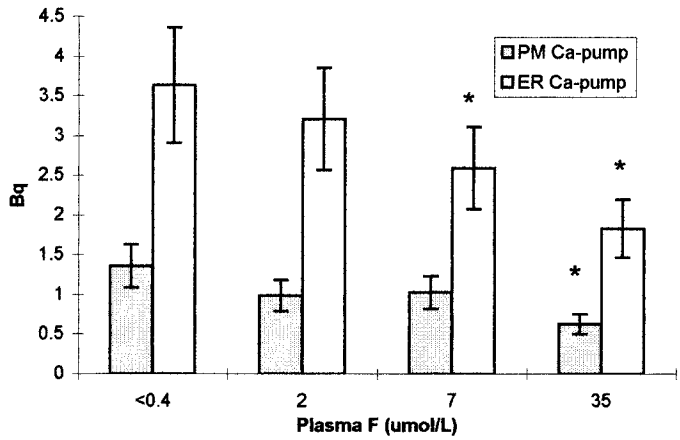

FIGURE 2 Plasma membrane Ca^{++}-pump (PM Ca-pump)-dependent uptake and thapsigargin-sensitive uptake representing endoplasmic reticulum Ca^{++}-pump (ER Ca-pump) -dependent uptake by kidney membrane vesicles from rats with plasma fluoride levels of <0.4, 2, 7, and 35 μmol F^-/L. Plasma fluoride concentrations were at these levels after 6 wk of consuming water containing 0, 10, 50, and 150 mg F^-/L, respectively. Values are means ± SD, n = 4–6 (see Table 1). *Significantly different ($P < 0.05$) from control (<0.4 μmol F^-/L).

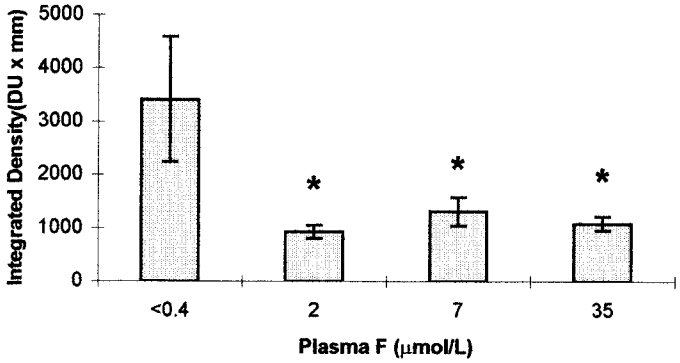

FIGURE 3 Quantification of the amount of plasma membrane Ca^{++}-pump protein in kidney membranes from rats with plasma fluoride levels of <0.4, 2, 7, and 35 μmol F^-/L. Plasma fluoride concentrations were at these levels after 6 wk of consuming water containing 0, 10, 50, and 150 mg F^-/L, respectively. Values are means ± SD, n = 4–6 (see Table 1). *Significantly different ($P < 0.05$) from control (<0.4 μmol F^-/L).

(Fig. 3; $P < 0.05$). Similar results were obtained for the endoplasmic reticulum Ca^{++}-pump **(Fig 4**; $P < 0.05$).

Western blot analysis of kidney membrane homogenates with monoclonal antibodies specific for Ca^{++}-pump proteins demonstrated the presence of single major bands at 135 kDa and 110 kDa representing the plasma membrane (PMCA) and endoplasmic reticulum (SERCA-2) Ca^{++}-pump proteins, respectively **(Fig. 5)**. The presence of lightly staining minor bands may represent breakdown products of the Ca^{++}-pumps. The possibility that these minor species represent additional proteins with low affinity binding or additional proteins in low abundance represents an alternative explanation. However, the overall contribution of these minor species to the colored reaction product appears very near background detection levels. This finding suggests that the colored product used for quantification via slot blot analysis is attributable to specific antibody binding to Ca^{++}-pump proteins.

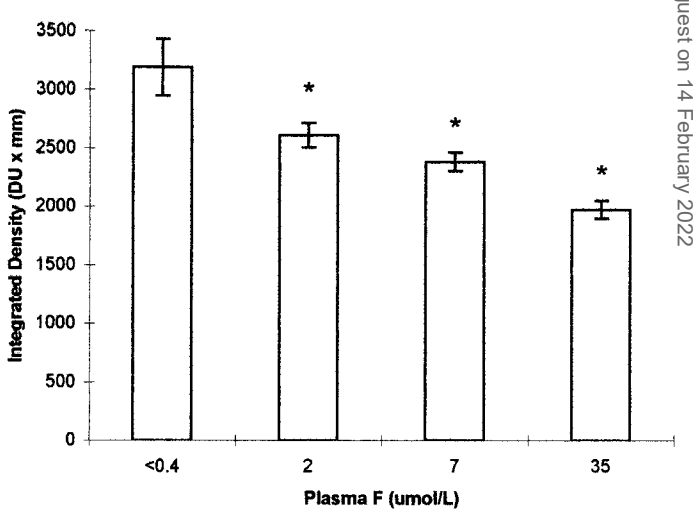

FIGURE 4 Quantification of the amount of endoplasmic reticulum Ca^{++}-pump protein in kidney membranes from rats with plasma fluoride levels of <0.4, 2, 7, and 35 μmol F^-/L. Plasma fluoride concentrations were at these levels after 6 wk of consuming water containing 0, 10, 50, and 150 mg F^-/L, respectively. Values are means ± SD, n = 4–6 (see Table 1). *Significantly different ($P < 0.05$) from control (<0.4 μmol F^-/L).

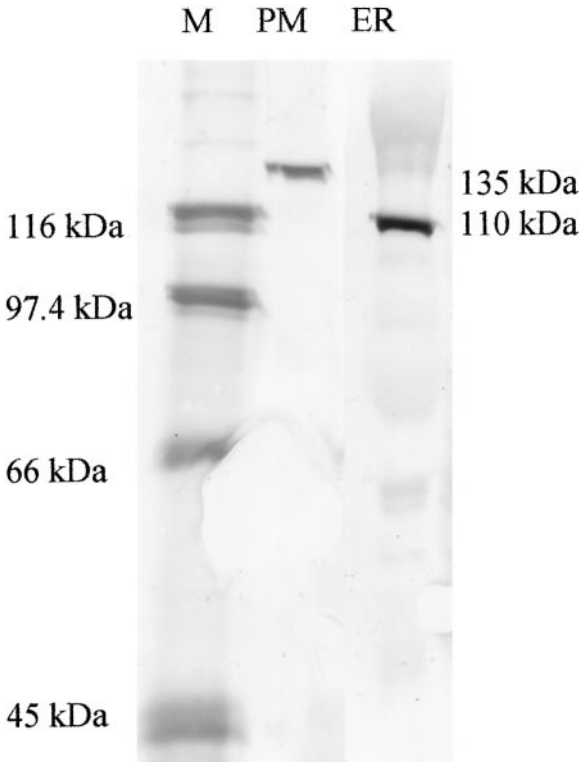

FIGURE 5 Western blot analysis of kidney membrane homogenates with antibodies specific for Ca^{++}-pump proteins. Marker proteins (M) of known molecular weight. Immunostaining with anti-plasma membrane Ca^{++}-pump (PM) and anti-endoplasmic reticulum Ca^{++}-pump (ER) monoclonal antibodies.

DISCUSSION

For most soft tissues, the tissue-to-plasma F^- ratios fall between 0.5 and 0.9. The exact tissue-to-plasma ratio for a given tissue depends on the magnitude of the transmembrane pH gradient, an observation consistent with the hypothesis that the highly permeating weak acid, HF (pK = 3.4), is in diffusion equilibrium across the membrane (Whitford 1996b). Tissue-to-plasma ratios for kidney, which are well above unity, represent a major exception to this generalization. The cortex-to-papilla concentration gradient for fluoride closely resembles that of sodium and chloride (Whitford and Taves 1973).

Although it was not demonstrated directly, it is also likely the fluoride concentrations among subcellular compartments depend on pH gradients. Thus, it would be expected that intracellular fluoride concentrations would be higher in mitochondria and nuclei in which the pH is higher than in the cytosol. We postulate, therefore, that this concentrating effect of the alkaline nuclear and mitochondrial fluids promotes the elevated fluoride concentrations necessary to affect the regulatory enzymes of the kidney.

A study by Narayanan et al (1991) showed both inhibitory and stimulatory effects of F^- on the Ca^{++}-pump of cardiac sarcoplasmic (endoplasmic). This Ca^{++}-pump, like the Ca^{++}-pump of the plasma membrane, transports Ca^{++} by a series of steps, including the binding of Ca^{++} ion; the phosphorylation of the enzyme by ATP to form a phosphorylated intermediate; and the subsequent conformational change in the enzyme, which results in the translocation of the Ca^{++} across the membrane. Following translocation of Ca^{++}, the phosphorylated enzyme intermediate breaks down, releasing Ca^{++}, after which the enzyme returns to its original conformational state for another cycle. The reported inhibitory and stimulatory effects of F^- on this process are explained by differing susceptibilities of the conformational states of the Ca^{++}-pump to the action of F^-. The susceptibility of the enzyme to F^- inhibition was postulated to occur in the presence or absence of Ca^{++}, but only for the dephosphorylated conformation of the enzyme. In these studies, inhibition occurred when F^- was added to the reaction prior to, but not excluding), the addition of ATP. When F^- was added after the ATP was added, a stimulatory effect was observed. This action appears similar to the known inhibitory action of vanadate on the plasma membrane Ca^{++} -pump and the Na^+-K^+ ATPase (Cantley et al. 1978, Inesi et al. 1984). The inhibition of these transporters occurs by the interaction of vanadate with the phosphorylation site of each molecule.

Studies by Holland (1979), and Dunapace and Stookey (1988), have shown that F^- inhibits both total protein and total DNA synthesis in cultured mammalian cells. In a recent review of the genetic toxicity of F^-, no apparent direct mechanism for F^- interaction with DNA was reported, (Zeiger et al. 1993). A more likely suggestion is that the inhibition of DNA synthesis may be a secondary effect of F^- interference with protein synthesis or as a result of the direct interaction of F^- with DNA polymerase or other enzymes associated with DNA synthesis, (Zeiger et al. 1993). Several studies have suggested that F^- can interact with divalent cations, such as Ca^{++}, in the cell, an affect which may disrupt enzyme systems necessary for DNA or RNA synthesis, or other cellular processes, (Edwards and Parry 1986, Harper et al. 1974, Hellung-Larsen and Klenow 1969, Holland 1979, Imai et al. 1983).

In our study we showed decreases in both the activity (Fig. 2) and amount (Figs. 3 and 4) of plasma membrane and endoplasmic reticulum Ca^{++}-pumps that corresponded to increases in plasma F^- levels, even when F^- was not added to the assay system. The observation that reductions in Ca^{++}-pump activity did not correlate directly with changes in the amount of Ca^{++}-pump protein suggests that additional factors that affect the activities of the enzymes may be involved. For example, there may be changes to the Ca^{++}-pump proteins that do not effect the antigenicity of the proteins. There may also be effects on the synthesis or degradation of other membrane proteins as well that affect ^{45}Ca uptake in our system.

Ca^{++} movement through the kidney tubules is controlled by Ca^{++} channels, Ca^{++}-binding proteins, Ca^{++}-sequestering organelles, Na^+/Ca^{++} exchangers, and Ca^{++}-pumps. Ca^{++} enters tubule cells through Ca^{++} channels down an electrical and chemical concentration gradient. Cytosolic Ca^{++} levels are kept low (near 10 μmol/L) by sequestration in organelles (for example, through the action of the endoplasmic reticulum Ca^{++}-pump) or through binding to calcium-binding proteins. Ca^{++} efflux from cells occurs by means of a Na^+/Ca^{++} exchanger or a plasma membrane Ca^{++}/Mg^{++} ATPase Ca^{++}-pump. Whereas both of these mechanisms function in the nephron of rats, the physiological role of the Na^+/Ca^{++} exchanger is unclear. Studies by Gmaj and Murer (1988) suggest that under normal physiological conditions, the Na^+/Ca^{++} exchanger may contribute only 10% as much to Ca^{++} efflux as the plasma membrane Ca^{++}-pump. Our studies used Na^+-free incubation conditions to eliminate the role of the Na^+/Ca^{++} exchanger in vesicle uptake.

In our study, a decrease in $^{45}Ca^{++}$ uptake was observed in kidney membranes isolated from rats after chronic F^- ingestion. The novel and important aspect of our study was that $^{45}Ca^{++}$ uptake was measured in uptake medium in the absence of added F^-. The decrease in Ca^{++} transport activity in these membranes was due to a decreased production of the

enzyme by the kidney cells, an increased turnover of the enzymes, or an increased breakdown of the enzymes by the cells. Stated differently, F^- appears to have affected the expression of the enzymes independent of any effect that F^- may have directly on the enzyme activity. Our study provides the first evidence that one of the effects of long-term F^- exposure is a change in expression of the plasma membrane and endoplasmic reticulum Ca^{++}-pumps in the kidney.

In summary, we provided rats with fluoride in their drinking water, which produced graded, plasma fluoride concentrations that occur in humans. Our studies showed that chronic high fluoride ingestion decreases the rate of Ca^{++} transport across renal tubule endoplasmic reticulum and plasma membranes, and reduced the amount of ER and PM Ca^{++}-pump protein present in the kidney membranes. We conclude that chronic high fluoride ingestion may decrease the expression, increase the breakdown, or increase the rate of turnover of plasma membrane and endoplasmic reticulum Ca^{++}-pump proteins and possibly other enzymes as well. The observed decreases in the rate of Ca^{++} transport and associated decreases in plasma membrane and endoplasmic reticulum Ca^{++}-pump expression could affect in vivo Ca^{++} homeostasis.

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