Sodium Fluoride Increases Intracellular Calcium in Rat Renal Epithelial Cell Line NRK-52E

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In our previous experiment using rats, fluoride was reported to cause renal calcification, whose mechanism was deduced to be due to an increase in parathyroid hormone (PTH) secretion. However fluoride-induced renal calcification that was independent of PTH has not been understood well in the nephron of fluoride-treated animals. Thus, we examined the effect of sodium fluoride on intracellular calcium mobilization in a normal rat kidney epithelial cell line (NRK-52E cells). The calcium accumulation was found to be remarkably increased by the addition of sodium fluoride (NaF). The elevation of $[Ca^{2+}]_i$ was demonstrated to be due to calcium entry through nifedipine-sensitive calcium channels. In addition, fluoride activates phospholipase C, but inositol 1,4,5-triphosphate (IP_3) didn't induce Ca^{2+} release from the endoplasmic reticulum (ER). Moreover, fluoride alone was deduced to enhance the activity of ER-type Ca^{2+} -ATPase.

Finally, on the mechanism of fluoride-induced calcium accumulation in NRK-52E cells, fluoride may activate phospholipase C to generate IP_3 and diacylglycerol, and these increases can be elucidated to induce calcium entry through dihydropyridine-sensitive calcium channels. Moreover, fluoride was found to stimulate calcium accumulation through ER-type Ca^{2+} -ATPase into the endoplasmic reticulum. The elevation of ER-type Ca^{2+} -ATPase activity by fluoride was elucidated to operate as a regulatory system to protect against abnormally higher increases in cytosolic calcium concentration via an increase of calcium influx into the endoplasmic reticulum.

Key words sodium fluoride; renal calcification; Ca influx; phospholipase C; inositol phosphate; diacylglycerol

Fluoride is considered an essential trace element, but is known to cause toxicity to animals or humans following acute or chronic exposure,^{1–5)} such as renal calcification, osteosclerosis, mottled teeth, glucosuria, electrolyte disturbance and so on. Chronic fluoride toxicity has been studied in livestock and dental fluorosis.^{6,7)} Experimentally-induced chronic toxicity in rodents is also associated with nephrotoxicity. In humans, acute toxicity usually occurs as a result of accidental or suicidal ingestion of fluoride, and it results in nephrotoxicity, gastrointestinal effects, severe hypercalcemia, hyperglycemia and shock.

In our previous experiment using rats, Suketa *et al.*, reported that fluoride causes renal calcification, whose mechanism was deduced as an increase in parathyroid hormone (PTH) secretion, and a subsequent large increase in renal calcium 24 h after the fluoride dose could be suppressed by thyroid-parathyroidectomy.⁸⁾ The subsequent large increase was demonstrated to be mainly due to the stimulation of parathyroid function as a secondary action of the fluoride dose.⁹⁾

PTH is known to regulate calcium reabsorption. With increased levels of PTH, there is increased calcium reabsorption, which reduces the urinary excretion of calcium. The major sites at which PTH stimulates renal calcium absorption are the renal cortical thick ascending loop of Henle^{10–13)} and the distal tubule.^{14,15)} In these nephron segments, PTH increases active transcellular calcium transport.¹⁶⁾ PTH which induces an increase in calcium absorption is associated with an increase of intracellular calcium activity ($[Ca^{2+}]_i$).

Active absorptive transport across polarized epithelial cells is a two-step process involving entry across apical plasma membranes followed by efflux across basolateral plasma membranes. Recently, Gesek reported that PTH increased calcium entry through dihydropyridine-sensitive calcium channels in mouse distal convoluted tubule cells.^{17,18)}

Calcium channels in electrically excitable cells such as cardiac myocytes or skeletal muscle cells are voltage-gated and activated at depolarizing membrane voltages.¹⁹⁾ In these experiments, it was revealed that PTH increases chloride conductance in renal distal tubule cells, leading to decreased intracellular chloride activity, membrane hyperpolarization, and increased calcium entry through dihydropyridine-sensitive calcium channels. However, such an effect of fluoride, which was independent of PTH, has been unknown in the nephron of fluoride-treated animals. Herein, we examined the effect of sodium fluoride (NaF) on $[Ca^{2+}]_i$ in normal rat kidney epithelial NRK-52E cells.

MATERIALS AND METHODS

Materials Dulbecco's modified Eagle's medium (D-MEM) and fetal bovine serum were obtained from Sigma (St. Louis, MO, U.S.A.). INDO 1-AM was obtained from Molecular Probes (Eugene, OR, U.S.A.). NaF and thapsigargin were purchased from Wako Pure Chemicals (Osaka, Japan), *myo*- $[^3H]$ inositol from NENTM Life Science Products (Boston, MA, U.S.A.), and Dowex-1 $\times$ 8 (100–200 mesh, chloride form) from Dow chemicals (Midland, MI, U.S.A.).

Cell Line and Culture The NRK-52E cells were isolated from normal rat kidney.²⁰⁾ The cells obtained from the American Type Culture Collection (ATCC CRL-1571), were cultured in D-MEM supplemented with 5% fetal bovine serum in an atmosphere of 5% CO_2 –95% air at 37 °C.

Measurement of $[Ca^{2+}]_i$ NRK-52E cells were grown to near confluence on glass coverslips (7 $\times$ 13 mm, thickness; under 0.12 mm, Matsunami Glass Ind., Osaka, Japan). Intracellular Ca^{2+} was measured with the Ca^{2+} probe INDO 1-AM, as previously described in detail.²¹⁾ In brief, the NRK-52E monolayers on the cover slips were rinsed 3 times with D-MEM phosphate buffered saline (PBS) buffer before incu-

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bation (10 μM INDO 1-AM, for at 37 °C in D-MEM-PBS). The composition of D-MEM-PBS was 5.55 mM glucose, 1.80 mM CaCl_2 , 5.36 mM KCl, 0.81 mM MgSO_4 , 109.51 mM NaCl, 0.84 mM NaH_2PO_4 , 30.83 mM NaHCO_3 , and 10.00 mM HEPES. The cover slips were mounted in a cuvette of a Hitachi F-2000 spectro-fluorometer in which temperature was controlled to 37 °C. INDO 1-loaded monolayers were perfused with D-MEM-PBS. The fluorescence of the dye was measured at an excitation wavelength of 330 nm and emission wavelength of 410 nm and 485 nm alternately. Absolute values of $[\text{Ca}^{2+}]_i$ were based on calibrations as described previously.²¹⁾

Cellular Uptake of $^{45}\text{Ca}^{2+}$ The uptake of $^{45}\text{Ca}^{2+}$ was performed as previously reported²¹⁾ with minor modifications. The 24-well clusters containing cell monolayers were fixed on a thermostatically controlled plate (37 °C). The monolayers were rinsed twice with D-MEM-PBS. NRK-52E cells were cultured in the presence of $^{45}\text{Ca}^{2+}$ (0.2 $\mu\text{Ci}/\text{well}$). Tracer uptake was linear for 10 min. After incubation time, the cells were washed 3 times at 0 °C with the following buffer: 140 mM NaCl, 4.7 mM KCl, 3.7 mM MgCl_2 , 2.0 mM LaCl_3 and 150 mM HEPES. The cells were dissolved with 500 μl of 0.5 N NaOH, and the radioactivity was measured by liquid scintillation counter. All experiments were normalized for protein content, which was measured by the Lowry method.²²⁾

Assay of Phospholipase C Activity Phospholipase C activity was assayed according to the production of inositol phosphate, such as inositol 1,4,5-triphosphate (IP_3), inositol 1,5-bisphosphate (IP_2) and inositol 1-monophosphate (IP_1). NRK-52E cells were cultured in the presence of *myo*- $[\text{^3H}]$ inositol (2 $\mu\text{Ci}/\text{ml}$) for 2 d. At 30 min before the experiments, cells were placed in a culture medium deprived of both *myo*- $[\text{^3H}]$ inositol and fetal calf serum. Cells were incubated in the following medium: 10 mM LiCl, 0.1 mM ATP, 0.25 M EDTA, 10 mM Tris-HCl (pH 8.0), 1 μM CaCl_2 , 5 mM MgCl_2 and 5 mM NaF. After indicated incubation times, cells were washed 3 times at 0 °C with PBS, scraped at 0 °C with a rubber policeman into the following medium: 10 mM LiCl, 0.1 mM ATP, 0.25 M EDTA, 10 mM Tris-HCl (pH 8.0), and homogenized at 4 °C in a Dounce Potter homogenizer equipped with a loose piston (11-fold). After rapid centrifugation (5 min, 1000 rpm), methanol/chloroform (2:1) was added in the supernatant. IP_1 , IP_2 and IP_3 present in the supernatant were separated by chromatography on Dowex-1 $\times$ 8 (formate form) columns (1.8 $\times$ 2.0 cm) by sequential elutions as described Berridge *et al.*²³⁾ with minor modifications.²⁴⁾ Labeled IP_1 , IP_2 and IP_3 were eluted by formic acid-ammonium formate buffers (0.1 M–0.2 M, 0.4 M and 1.0 M, respectively). Radioactivity found in the inositol phosphates was measured by a liquid scintillation counter.

Statistical Analysis The statistical significance of differences between the groups was determined by applying the Student's *t*-test.

RESULTS

Fluoride Increases Calcium Accumulation in NRK-52E Cells PTH increased calcium accumulation in primary cell cultures of distal tubules and cortical thick limb cells.^{10–15)} However, a direct effect of fluoride has been unknown. The

Fig. 1. NaF Increases Calcium Accumulation in NRK-52E Cells

NRK-52E cells were cultured as described in Materials and Methods and $^{45}\text{Ca}^{2+}$ uptake was measured as described in Materials and Methods. Cells treated with a range of 0.3–30 mM NaF were incubated in a medium containing $^{45}\text{Ca}^{2+}$. Data are expressed as a percent of the control (calculated from four experiments) and standard error as a bar. * $p < 0.01$, ** $p < 0.005$, significantly different from the control.

Fig. 2. Nifedipine Suppresses Increment of Calcium Accumulation in NRK-52E Cells Treated by NaF

NRK-52E cells were cultured as described in Materials and Methods and $^{45}\text{Ca}^{2+}$ uptake was measured as described in Materials and Methods. Cells treated with 10 mM NaF were exposed for 10 min in the absence or presence of 20 μM nifedipine. Data are expressed as a percent of the control (calculated from 4 experiments) and standard error as a bar. * $p < 0.01$, significantly different from the absence of NaF, ** $p < 0.01$, significantly different from the presence of NaF.

effects of fluoride on calcium accumulation in NRK-52E cells are illustrated in Fig. 1. Data are expressed as a percent of the control. Fluoride effectively increases $^{45}\text{Ca}^{2+}$ uptake in NRK-52E cells. The calcium uptake was increased to 6.38-fold of the control by 10 mM sodium fluoride. The 1,4-dihydropyridine calcium channel antagonist, nifedipine (20 μM), suppressed the fluoride-induced increase of $^{45}\text{Ca}^{2+}$ uptake, as shown in Fig. 2. These results support the view that fluoride-induced increase of calcium entry in NRK-52E cells is mediated by calcium channels. Nifedipine had no inhibitory action of its own, as shown in Fig. 2.

Fluoride Increases Calcium Entry from Extracellular Ca^{2+} through Dihydropyridine-Sensitive Calcium Channels in NRK-52E Cells To confirm the calcium entry pathway in NRK-52E cells, we examined the time course of $[\text{Ca}^{2+}]_i$ using a Ca^{2+} probe, INDO 1. The $[\text{Ca}^{2+}]_i$ was determined in 4 experiments and the results are illustrated in Fig. 3. Fluoride effectively increased $[\text{Ca}^{2+}]_i$ in NRK-52E cells (Fig. 3A), and this increased $[\text{Ca}^{2+}]_i$ entry from extracellular Ca^{2+} (Fig. 3B). These results confirm that fluoride-induced increases in calcium entry are not mediated by an IP_3 recep-

tor on endoplasmic reticulum, but from calcium channels at the plasma membrane. Nifedipine (20 μM) suppressed the fluoride-induced $[\text{Ca}^{2+}]_i$ increase effectively (Fig. 3C), but verapamil (20 μM) had almost no effect on fluoride-induced $[\text{Ca}^{2+}]_i$ elevation (Fig. 3D), therefore this fluoride-induced increase in calcium entry was through dihydropyridine-sensitive, verapamil-insensitive calcium channels.

Fluoride Increases Phospholipase C Activity Phospholipase C activity was assayed as inositol phosphate (IP_3 , IP_2 and IP_1) accumulation. Figure 4 shows inositol phosphate accumulation in the incubation medium. Membranes prepared from NRK-52E cells were grown in the presence of *myo*- $[\text{^3H}]$ inositol (2 $\mu\text{Ci}/\text{ml}$). Fluoride effectively increased the accumulation of IP_3 and IP_2 (Fig. 4A and B). IP_3 accumulation in the presence of 10 mM sodium fluoride for 6 min and 12 min reached to 5.12-fold and 6.34-fold of the control

level, respectively. IP_2 accumulation in the presence of 10 mM sodium fluoride for 6 min and 12 min was 7.01-fold and 8.74-fold that of the control, respectively. On the other hand, IP_1 did not change and/or slightly declined (Fig. 4C). This may have resulted from a slow inositol phosphate breakdown during the process of membrane preparation. These observations suggest that the activity of phospholipase C in the membrane is stimulated effectively by fluoride.

Fluoride Increases Calcium Entry through ER-type Ca^{2+} -ATPase into Endoplasmic Reticulum To confirm the mechanism of calcium accumulation in NRK-52E cells, we examined the change in $[\text{Ca}^{2+}]_i$ with or without thapsigargin (TG). TG is a specific inhibitor of ER-type Ca^{2+} -ATPase, and is much less effective on cardiac SR-type Ca^{2+} -ATPase and apparently ineffective against enzymes from sarcoplasmic reticulum and plasma membrane.²⁵ TG alone had no effect on $[\text{Ca}^{2+}]_i$, but fluoride more effectively increased $[\text{Ca}^{2+}]_i$ in the presence of TG than in its absence (Fig. 5). This increase in $[\text{Ca}^{2+}]_i$ in the presence of TG is thought to be due to blocking a stimulation of calcium influx through ER-type Ca^{2+} -ATPase into endoplasmic reticulum induced by fluoride. This result suggests that fluoride activates ER-type Ca^{2+} -ATPase.

DISCUSSION

According to many observations, fluoride induces PTH-mediated renal calcification.^{8,9} Whether fluoride directly causes calcium accumulation has been unknown. In this paper, we first examined the direct effect of fluoride on calcium accumulation in normal rat kidney epithelial NRK-52E cells, and then attempted to clarify the mechanism of the calcium accumulation by fluoride. The homeostasis of $[\text{Ca}^{2+}]_i$ is determined to induce active fluxes of calcium entry and calcium extrusion, as well equilibrium between free Ca^{2+} and Ca^{2+} -binding proteins.^{26,27} Calcium entry generally occurs through calcium channels in the plasma membrane or through IP_3 -gated release from the endoplasmic reticulum.

We observed that NaF induces both calcium accumulation in NRK-52E cells (Fig. 1), and elevation of $[\text{Ca}^{2+}]_i$ through dihydropyridine-sensitive calcium channels (Figs. 3C and D). Next, we examined the relationship between ER-type Ca^{2+} -

Fig. 3. NaF Increases Intracellular Calcium ($[\text{Ca}^{2+}]_i$) in NRK-52E Cells

Measurement of $[\text{Ca}^{2+}]_i$ was described in Materials and Methods. Cells were grown on cover slips and loaded with the Ca^{2+} probe INDO 1-AM. Data show the effect of 10 mM sodium fluoride in the presence of extracellular Ca^{2+} (A), or in the absence of extracellular Ca^{2+} containing 500 μM EGTA (B). Data show the effect of 10 μM NaF in the presence of 20 μM nifedipine (C), or in the presence of 20 μM verapamil (D). These results are averaged from a total of 4 experiments.

Fig. 4. Effect of Fluoride on Phospholipase C Activity in NRK-52E Cells

Phospholipase C activity was assayed as a production of inositol phosphates, such as IP_3 (A), IP_2 (B) and IP_1 (C). Membranes prepared from NRK-52E cells were grown 2 d in the presence of *myo*- $[\text{^3H}]$ inositol (2 $\mu\text{Ci}/\text{ml}$), and then inositol phosphates were determined as indicated in Materials and Methods. Data are expressed as a percent of the control (calculated from 4 experiments) and standard errors as bars. * $p < 0.01$, significantly different from the absence of NaF.

Fig. 5. Thapsigargin Increases $[Ca^{2+}]_i$ in NRK-52E Cells

Measurement of $[Ca^{2+}]_i$ was described in Materials and Methods. Cells were grown on cover slips and loaded with the Ca^{2+} probe INDO 1-AM. Data show the effect of 10 mM NaF in the presence (upper trace) or absence of thapsigargin (TG) (lower trace). These results are averaged from a total of 4 experiments.

ATPase and the elevation of $[Ca^{2+}]_i$ using TG, which is a specific inhibitor of the enzyme. From the results, fluoride was found to increase calcium entry through ER-type Ca^{2+} -ATPase into endoplasmic reticulum (Fig. 5). These results suggested that the cytosolic calcium increase by fluoride was due to an elevation of influx from extracellular calcium by calcium channel gate-opening, but not to calcium efflux from the endoplasmic reticulum.

IP_3 is a key second messenger, the primary function of which is to elevate $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from the endoplasmic reticulum. With secondary activation of Ca^{2+} influx at the plasma membrane, Guillon *et al.* found that phospholipase C is activated by fluoride in WRK-1 cell membranes.²⁴⁾ Thus, we determined the effect of fluoride on phospholipase C activity in NRK-52E cell membranes. Fluoride effectively increases the accumulation of IP_3 and IP_2 (Figs. 4A and B). These observations demonstrated that phospholipase C localizes in this membrane preparation using PIP_2 as a substrate, and that fluoride effectively activates phospholipase C in NRK-52E cells. Tshipamba reported the Ca dependence of IP_3 -induced Ca^{2+} release in porcine renal epithelial LLC-PK₁ cells.²¹⁾ Davis *et al.* reported the IP_3 -mediated cytosolic and nuclear calcium elevation in NRK-52E cells using caged glycerophosphoryl- PIP_2 .²⁸⁾ Interestingly, our results suggested that IP_3 didn't induce calcium entry through IP_3 -gated release from the endoplasmic reticulum (Fig. 3B). These results suggest that fluoride-induced IP_3 may be involved in the activation of calcium channels at the plasma membrane, but not with IP_3 receptor at the endoplasmic reticulum in NRK-52E cells.

Our results, of course, cannot entirely explain the mechanism involved in calcium accumulation induced by fluoride.^{29,30)} Another product of phospholipase C is diacylglycerol, which activates protein kinase C-mediated calcium channels at the plasma membrane. Diacylglycerol is also known to be supplied from phosphatidylcholine by phospholipase C. The hydrolysis of phosphatidylcholine has been reported to be enhanced by insulin-like growth factor, and epithelial growth factor.^{31,32)} These growth factors directly activate calcium channels and concurrently induce the hydrolysis of phosphatidylcholine to generate diacylglycerol. In this regard, diacylglycerol may be a key point in the elevation of $[Ca^{2+}]_i$ by fluoride.

Finally, regarding the mechanism of fluoride-induced calcium accumulation in NRK-52E cells, it could be deduced that fluoride activates phospholipase C to increase IP_3 and diacylglycerol, and these lead to increase calcium entry through dihydropyridine-sensitive calcium channels. Moreover, the elevation of ER-type Ca^{2+} -ATPase activity by fluoride was elucidated to operate as a regulatory system to protect against abnormally high increases in cytosolic calcium concentration *via* increases in calcium influx into the endoplasmic reticulum.

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