

Original Article

# Ca<sup>2+</sup> entry through reverse Na<sup>+</sup>/Ca<sup>2+</sup> exchanger in NCI-H716, glucagon-like peptide-1 secreting cells

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**ABSTRACT** Glucagon like peptide-1 (GLP-1) released from enteroendocrine L-cells in the intestine has incretin effects due to its ability to amplify glucose-dependent insulin secretion. Promotion of an endogenous release of GLP-1 is one of therapeutic targets for type 2 diabetes mellitus. Although the secretion of GLP-1 in response to nutrient or neural stimuli can be triggered by cytosolic Ca<sup>2+</sup> elevation, the stimulus-secretion pathway is not completely understood yet. Therefore, the aim of this study was to investigate the role of reverse Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (rNCX) in Ca<sup>2+</sup> entry induced by muscarinic stimulation in NCI-H716 cells, a human enteroendocrine GLP-1 secreting cell line. Intracellular Ca<sup>2+</sup> was repetitively oscillated by the perfusion of carbamylcholine (CCh), a muscarinic agonist. The oscillation of cytosolic Ca<sup>2+</sup> was ceased by substituting extracellular Na<sup>+</sup> with Li<sup>+</sup> or NMG<sup>+</sup>. KB-R7943, a specific rNCX blocker, completely diminished CCh-induced cytosolic Ca<sup>2+</sup> oscillation. Type 1 Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX<sub>1</sub>) proteins were expressed in NCI-H716 cells. These results suggest that rNCX might play a crucial role in Ca<sup>2+</sup> entry induced by cholinergic stimulation in NCI-H716 cells, a GLP-1 secreting cell line.

## INTRODUCTION

Glucagon-like peptide-1 (GLP-1) secreted from intestinal enteroendocrine L-cells is an incretin that can augment glucose-dependent insulin secretion [1]. GLP-1 is known to stimulate proinsulin gene expression and pancreatic  $\beta$ -cell proliferation [2,3]. Promoting insulin secretion is a crucial goal when treating type 2 diabetes mellitus. Thus, stimulating endogenous release of GLP-1 has become one of therapeutic targets for diabetes [4,5]. A number of cellular models, such as isolated primary intestinal L-cell and enteroendocrine cell lines STC-1, GLUTag, and NCI-H716 cells, have been used to study the regulation of GLP-1 secretion [6,7]. These cellular models provide useful information concerning signaling pathways that regulate GLP-1 secretion. NCI-H716 cells derived from poorly differentiated adenocarcinoma of human cecum have potent GLP-1 secreting properties. Nutrients, hormones, and neurotransmitters are known to control the release of

GLP-1 in physiological conditions [8,9]. It has been reported that muscarinic agonist can stimulate GLP-1 secretion in NCI-H716 cells, and that such secretion is closely linked to G-protein coupled receptor activations and elevated cytosolic Ca<sup>2+</sup> signals [10-12]. Although GLP-1 secretion plays an important role in blood glucose control as mentioned above, the detail stimulus-secretion pathway in NCI-H716 cells is not completely understood yet.

Mammalian Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX) is a bi-directional Ca<sup>2+</sup> transporter that contributes to Ca<sup>2+</sup> homeostasis of multiple tissues such as cardiac muscle, skeletal muscle, brain, and kidney [13]. Mammalian cells are known to express three types of NCX isoforms [14,15]. NCX1 is broadly expressed in various cells. It is particularly abundant in the heart, brain, and kidney. However, NCX2 is locally expressed in the brain and NCX3 is limitedly expressed in the brain and skeletal muscles. The direction of Ca<sup>2+</sup> movement depends on electrical and chemical gradients across the membrane. The forward mode of NCX generates an inward



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Na<sup>+</sup> current and an outward Ca<sup>2+</sup> current while the reverse-mode NCX promotes Na<sup>+</sup> efflux and Ca<sup>2+</sup> influx in plasma membrane. It is usually accepted that the forward NCX is favored at resting membrane potential and that reverse NCX (rNCX) may operate when the cell is physiologically depolarized [13-16]. Na<sup>+</sup> dependency of GLP-1 secretion has been suggested because glucose-induced GLP-1 secretion can be blocked by replacing extracellular Na<sup>+</sup> with *N*-methyl-D-glucamine (NMG<sup>+</sup>) in GLUTag and intestinal L-cells [7,17]. Since the secretion of GLP-1 in response to nutrient or neural stimulation can be triggered by elevation of cytosolic Ca<sup>2+</sup>, extracellular Na<sup>+</sup> may be involved in the stimulus-secretion pathway of NCI-H716 cells. Therefore, the purpose of this study was to investigate the existence and the role of reverse Na<sup>+</sup>/Ca<sup>2+</sup> exchanger on Ca<sup>2+</sup> mobilization under cholinergic stimulation in NCI-H716 cells. Here we report that NCX1 is present on the plasma membrane and that rNCX contributes to calcium entry for calcium oscillation under cholinergic stimulation in NCI-H716, an enteroendocrine GLP-1 secreting cell line.

## METHODS

### Culture of NCI-H716 cells

Human enteroendocrine NCI-H716 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained as a suspension culture in RPMI 1640 at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> as described previously [18]. Two days before experiments, endocrine differentiation and cell adhesion cells were initiated by seeding cells onto cover glass in 6-well culture plates coated with Matrigel (Becton Dickinson Co., Bedford, MA, USA) in high-glucose DMEM. On the day of experiments, to stabilize cultured cells and measure cytosolic Ca<sup>2+</sup>, cells were resuspended with HEPES-buffered physiological saline containing 137 mM NaCl, 4.7 mM KCl, 0.56 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.28 mM CaCl<sub>2</sub>, 10 mM HEPES, and 5.5 mM glucose (pH 7.4).

### Intracellular Ca<sup>2+</sup> measurements

Cultured cells were loaded with 5 μM Fura-2/AM, a low affinity Ca<sup>2+</sup> sensitive dye, for 30 min at room temperature to measure intracellular Ca<sup>2+</sup>. Fura-2/AM loaded cells were continuously perfused with HEPES-buffered physiological saline. The perfusion rate was consistently controlled to be 1 ml/min using an electronically controlled perfusion system (Warner Instrument, Hamden, CT, USA). Intracellular Ca<sup>2+</sup> levels were monitored using a TILL Photonics imaging system (Till photonics, Pleasanton, CA, USA). Fura-2/AM loaded cells were excited alternately with light at 340 nm and 380 nm using a Polychrome V monochromator (TILL Photonics). Fluorescence images emitted at 510 nm through 40× fluorescence objective lens were captured using a Cool-SNAP

HQ<sub>2</sub> camera (Photometrics, Tuscon, AZ, USA) attached to an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan) [19].

### Western blots and immunocytochemistry

Protein samples were prepared from cultured NCI-H716 cells. Briefly, cells were pelleted and homogenized in an ice-cold lysis buffer containing 50 mM Tris/HCl, 2 mM EDTA, 10 mM EGTA, 5 mM DTT, 250 mM sucrose, 1% Triton X-100, and protease inhibitors (pH 7.5). Protein concentrations were measured using Bradford's protein assay reagents (Bio-Rad Laboratories, Hercules, CA, USA). Samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane at room temperature. After overnight incubation with polyclonal NCX1 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C, membranes were then incubated with goat anti-rabbit IgG horseradish peroxidase secondary antibodies for 1 h at 37°C. Bands were visualized using enhanced chemiluminescence. To determine distributions of NCX1, prepared NCI-H716 cells were placed on a poly-L-lysine coated glass coverslip for 10 min at room temperature prior to fixation with 2% formaldehyde in PBS solution. After fixation, nonspecific bindings were blocked by 1 h incubation in 3% BSA medium. The immunocytochemistry was performed using a primary NCX1 antibody and Cy-2 goat anti-rabbit secondary antibody. Immunofluorescence images for NCX1 were collected with an Olympus IX71 inverted microscope.

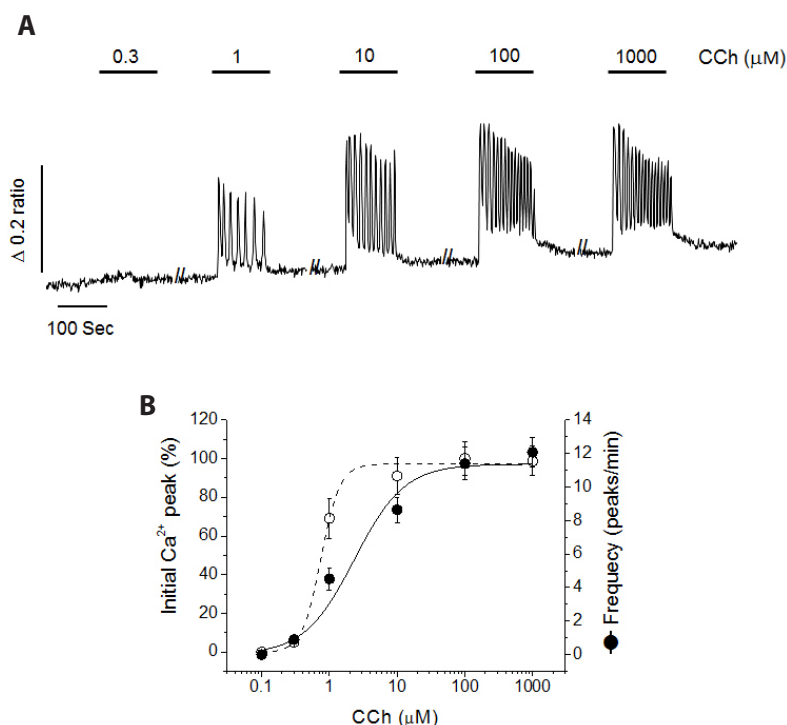
### Data analysis

Change of cytosolic Ca<sup>2+</sup> was expressed as a representative trace in individual cells. Summated results were presented as mean ± SE. Differences were considered significant when the p-value was less than 0.05 using Student's *t*-test.

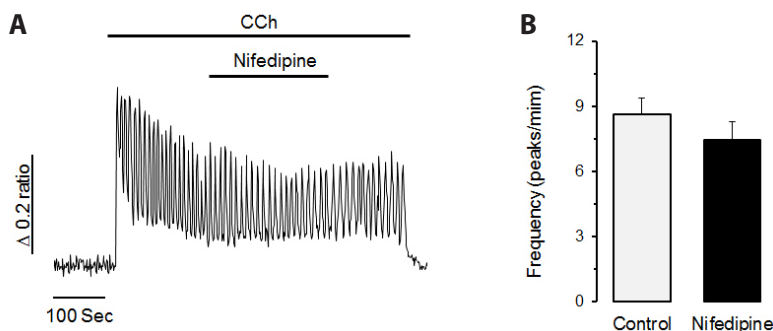
## RESULTS

### Cholinergic stimulation induced cytosolic Ca<sup>2+</sup> oscillation

Initial experiments were performed to investigate if cholinergic stimuli could generate oscillatory cytosolic Ca<sup>2+</sup> signals in NCI-H716 cells. Changes of intracellular Ca<sup>2+</sup> concentration were monitored using various concentrations of carbamylcholine (CCh, 0.3 μM–1 mM), a long-acting acetylcholine analog in the presence of 1.28 mM extracellular Ca<sup>2+</sup>. As shown in Fig. 1A, the perfusion above 1 μM of CCh for 100 sec evoked an initial Ca<sup>2+</sup> peak. Oscillatory Ca<sup>2+</sup> signals then continued. The maximum effect of initial Ca<sup>2+</sup> peak was observed at 100 μM of CCh. Half maximum dosage of initial Ca<sup>2+</sup> peak was 0.85 ± 0.09 μM of CCh. The maximum frequency of Ca<sup>2+</sup> oscillation was 12.02 ± 0.89 peaks/min. Half maximum effect was observed at 2.98 ± 0.47 μM of CCh (Fig.



**Fig. 1. Dose-dependent Ca<sup>2+</sup> oscillation induced by carbamylcholine (CCh) in NCI-H716 cells.** (A) Representative cytosolic Ca<sup>2+</sup> oscillation obtained from stepwise increase of various concentration of CCh (0.3 μM–1 mM) every 100 sec. (B) CCh-induced initial Ca<sup>2+</sup> peak (% of maximum) and frequency of Ca<sup>2+</sup> oscillation (peaks/min). Cytosolic Ca<sup>2+</sup> measurement was obtained from seven separate experiments in fura-2 loaded NCI-H716 cells. CCh significantly stimulated initial Ca<sup>2+</sup> peak and frequency of Ca<sup>2+</sup> oscillation, dose-dependently.

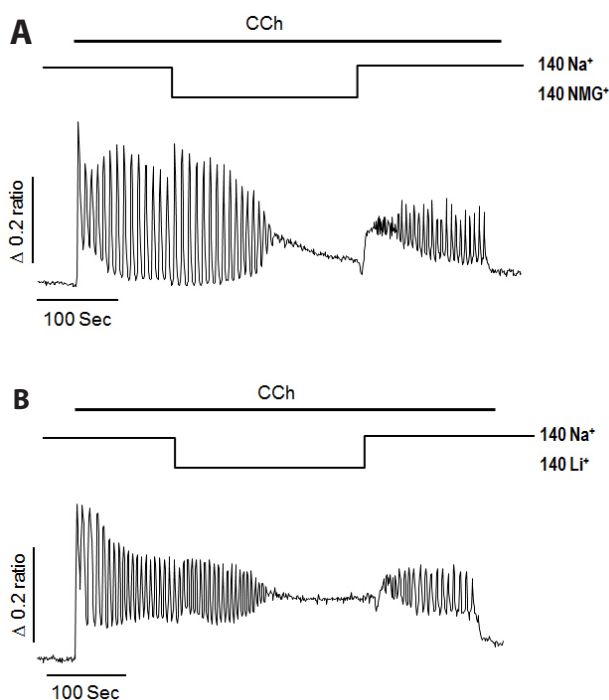


**Fig. 2. Voltage-operated Ca<sup>2+</sup> channel (VOC) does not contribute to carbamylcholine (CCh)-induced Ca<sup>2+</sup> entry process in NCI-H716 cells.** (A) Nifedipine, a VOC antagonist, failed to change CCh-induced Ca<sup>2+</sup> oscillation in NCI-H716 cells. (B) CCh-induced frequency of Ca<sup>2+</sup> oscillation was not changed by nifedipine treatment. Similar results were obtained from six separate experiments in NCI-H716 cells. VOC might not be involved in the calcium influx in CCh-stimulated NCI-H716 cells.

1B). These results suggest that cholinergic stimulation could sufficiently generate oscillatory Ca<sup>2+</sup> signals in NCI-H716 cells. Therefore, we used CCh at a concentration of 10 μM in the following experiments to generate repetitive Ca<sup>2+</sup> oscillation. The following experiment was performed to determine whether voltage-operated Ca<sup>2+</sup> channel (VOC) was involved in CCh-induced calcium oscillation in NCI-H716 cells. As shown in Fig. 2A and 2B, Ca<sup>2+</sup> oscillation signal induced by CCh was not suppressed by nifedipine, a VOC blocker. These results indicate that VOC does not participate in CCh-stimulated calcium influx in NCI-H716 cells.

### Elimination of extracellular Na<sup>+</sup> terminates CCh-induced Ca<sup>2+</sup> oscillation

Next, we determined whether Na<sup>+</sup>/Ca<sup>2+</sup> exchanger could contribute to CCh-induced oscillatory Ca<sup>2+</sup> signals in NCI-H716 cells. Preferentially, we eliminated extracellular Na<sup>+</sup> by replacing Na<sup>+</sup> with NMG<sup>+</sup> or Li<sup>+</sup>. As shown in Fig. 3A, the replacement of Na<sup>+</sup> with NMG<sup>+</sup> resulted in a slight enhancement of Ca<sup>2+</sup> oscillation pattern in the initial state, followed continuous decreases. Cytosolic Ca<sup>2+</sup> oscillation was completely terminated at about 120 sec af-



**Fig. 3. Effects of extracellular free  $\text{Na}^+$  on carbamylcholine (CCh)-induced  $\text{Ca}^{2+}$  oscillation in NCI-H716 cells.** (A) Substitution of  $\text{Na}^+$  with *N*-methyl-D-glucamine ( $\text{NMG}^+$ ) resulted in cessation of  $\text{Ca}^{2+}$  oscillation, which was restored by  $\text{Na}^+$  reperfusion. (B) Replacing  $\text{Na}^+$  with  $\text{Li}^+$  resulted in termination of cytosolic  $\text{Ca}^{2+}$  oscillation and restoration of oscillatory  $\text{Ca}^{2+}$  signals by  $\text{Na}^+$  reperfusion.  $\text{Na}^+$ -dependent  $\text{Ca}^{2+}$  influx might be important for the generation of oscillatory  $\text{Ca}^{2+}$  signaling induced by muscarinic stimulation in stimulus-secretion mechanism of NCI-H716 cells.

ter  $\text{Na}^+$  elimination. Reintroduction of extracellular  $\text{Na}^+$  resulted in a slow restoration of oscillatory  $\text{Ca}^{2+}$  signals. When  $\text{Na}^+$  was replaced with  $\text{Li}^+$ , cytosolic  $\text{Ca}^{2+}$  oscillation was slowly terminated similar to the replacement of  $\text{Na}^+$  with  $\text{NMG}^+$ . Such termination was then also partially recovered by  $\text{Na}^+$  reperfusion (Fig. 3B). these data indicate that extracellular  $\text{Na}^+$  may be essential to  $\text{Ca}^{2+}$  influx for the generation of oscillatory  $\text{Ca}^{2+}$  signaling induced by muscarinic stimulation in the stimulus-secretion mechanism of NCI-H716 cells. Thus, the following experiment was planned to evaluate whether KB-R7943, a specific reverse-mode NCX blocker, could affect CCh-induced oscillatory  $\text{Ca}^{2+}$  signaling in NCI-H716 cells.

### Effects of KB-R7943 on CCh-induced $\text{Ca}^{2+}$ oscillation

Addition of KB-R7943 remarkably abolished 10  $\mu\text{M}$  CCh-induced  $\text{Ca}^{2+}$  oscillation. Such abolishment was recovered by withdrawing KB-R7943 (Fig. 4A). Similar results were observed when extracellular medium was changed to free  $\text{Ca}^{2+}$  buffer (Fig. 4B). In contrast, pretreatment of KB-R7943 had no obvious effect on the initial  $\text{Ca}^{2+}$  peak (Fig. 4C, D). Indeed, cholinergic stimuli known to generate initial cytosolic  $\text{Ca}^{2+}$  peaks with sustained os-

illatory  $\text{Ca}^{2+}$  signals. Initial  $\text{Ca}^{2+}$  peaks are originated by inositol 1,4,5-trisphosphate-dependent calcium release from intracellular calcium stores and sustained  $\text{Ca}^{2+}$  oscillation in need of calcium influx from extracellular medium in secretory cells [20,21]. In the present study, KB-R7943 reduced only maintenance of oscillatory  $\text{Ca}^{2+}$  signaling without affecting initial  $\text{Ca}^{2+}$  transients. These results lead us to conclude that rNCX might contribute to the generation of CCh-induced cytosolic  $\text{Ca}^{2+}$  oscillation by modulating  $\text{Ca}^{2+}$  influx pathways from extracellular fluid in NCI-H716 cells.

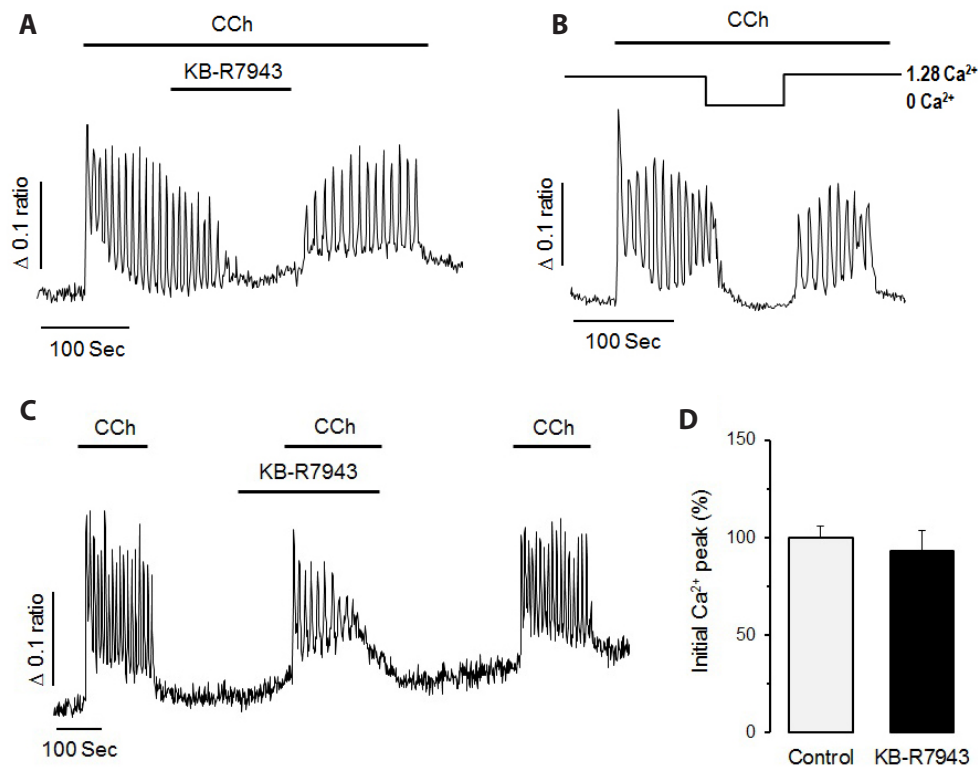
### Expression of NCX1 proteins in NCI-H716 cells

In order to determine whether NCI-H716 cells could express NCX1 at the protein level, we performed Western blot analysis from prepared cell lysates using commercial primary antibodies to NCX1. NCX1 protein was detected in NCI-H716 cells at a band of about 120 kDa (Fig. 5A). Additional band of a smaller size at 67 kDa probably represent proteolytic product of NCX protein. Such degradation product of NCX has well described in other tissues [22]. In the immunocytochemical experiment on NCX1 protein localization, as shown in Fig. 5B, NCX1 protein was expressed on NCI-H716 cells. There was no non-specific background staining in the negative experiment without primary antibody.

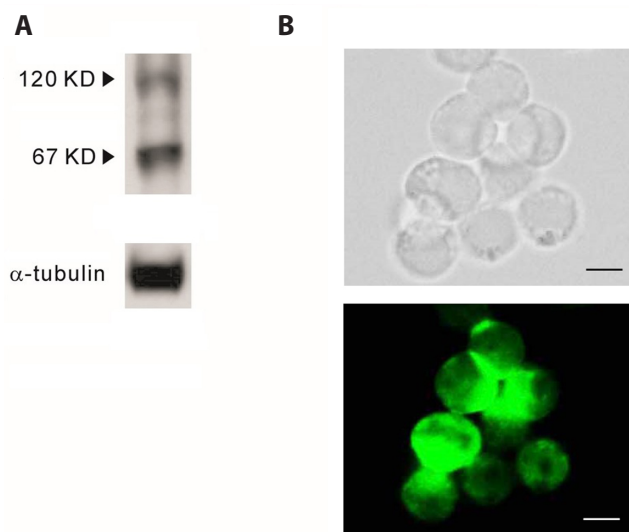
## DISCUSSION

The present study provides evidence that reverse-mode NCX might significantly contribute to acetylcholine-induced  $\text{Ca}^{2+}$  entry pathway of NCI-H716, a GLP-1 secreting cell line. Since GLP-1 is a well-known incretin hormone, many studies have been focused on stimulus-secretion mechanism of GLP-1 secreting cells to develop therapeutics for diabetes mellitus. In general, muscarinic receptors are closely linked to G-protein coupled receptor activation [10-12]. It is already known that muscarinic activation can enhance the secretion of GLP-1 in NCI-H716 cells [23]. Thus, muscarinic agonists can mobilize  $\text{Ca}^{2+}$  from internal stores through activation of  $\text{InsP}_3$  receptors, which subsequently activates store-operated  $\text{Ca}^{2+}$  entry from the external medium to refill depleted stores in NCI-H716 cells, similarly to other non-excitable epithelial cells [20,21]. However, these issues have not yet been addressed in detail. In this study, we confirmed that CCh could effectively generate oscillatory  $\text{Ca}^{2+}$  signals in NCI-H716 cells. The initial  $\text{Ca}^{2+}$  peak and the rhythmic  $\text{Ca}^{2+}$  frequency induced by CCh were also increased in a dose-dependent manner. These results support that muscarinic receptor-mediated  $\text{Ca}^{2+}$  signaling pathways which may act as a critical mechanism for regulating GLP-1 secretion are well operated in NCI-H716 cells.

In this study, for the first time, rhythmic calcium oscillation was observed in NCI-H716 cells by cholinergic stimulation. In general, repetitive and regular calcium oscillation can help intermittent message to include various information as the frequency,



**Fig. 4. Effects of KB-R7943 on carbamylcholine (CCh)-induced Ca<sup>2+</sup> oscillation in NCI-H716 cells.** (A) KB-R7943 significantly blocked CCh-induced Ca<sup>2+</sup> oscillation in NCI-H716 cells. Ca<sup>2+</sup> oscillation was restored after cessation of KB-R7943 perfusion. (B) Elimination of extracellular Ca<sup>2+</sup> resulted in complete inhibition of CCh-induced Ca<sup>2+</sup> oscillation. (C) Pretreatment of KB-R7943 failed to inhibit initial Ca<sup>2+</sup> peak induced by CCh. (D) Effect KB-R7943 on CCh-induced initial Ca<sup>2+</sup> peak obtained from seven separate experiments. rNCX might contribute to the generation of CCh-induced cytosolic Ca<sup>2+</sup> oscillation by modulating Ca<sup>2+</sup> influx pathway from extracellular fluid in NCI-H716 cells.



**Fig. 5. Type 1 Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX1) protein expression and distribution in NCI-H716 cells.** (A) Western blot analysis of NCX1 expression. NCX1 protein was detected in NCI-H716 cells at band of about 120 kDa, and small sized proteolytic product band of 67 kDa was additionally detected. (B) Immunocytochemistry for NCX1 protein expression. Corresponding bright-field (top) and immunofluorescence (bottom) microscopy images showing the labelling of NCX1 protein. NCX1 protein was remarkably expressed on NCI-H716 cells, scale bars = 10 μm.

of GLP-1. It is not yet clear which types of calcium channels are involved in changes in intracellular calcium concentration in NCI-H716 cells. Some studies have reported that depolarization of cell membrane in GLP-1 secreting cells, such as GLUTag cells or primary intestinal cells, can promote the secretion of GLP-1 [26,27]. However, the oscillatory calcium signal induced by CCh was unaffected by voltage-operated Ca<sup>2+</sup> channel blocker nifedipine in this study. These results suggest that voltage-operated Ca<sup>2+</sup> channels do not participate in CCh-induced oscillatory calcium signals in NCI-H716 cells. Recently, sodium dependency on GLP-1 secretion in intestinal L-cells has been suggested because glucose-induced GLP-1 secretion can be blocked by replacement of extracellular Na<sup>+</sup> with NMG<sup>+</sup> [7,17]. The present study also confirmed that when Na<sup>+</sup> in the extracellular fluid was removed by substituting NMG<sup>+</sup> or Li<sup>+</sup>, CCh-induced calcium oscillation disappeared. These results suggest that the calcium oscillation signal generated in NCI-H716 cells has a sodium dependence.

Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX), a bi-directional Ca<sup>2+</sup> transporter, is known to contribute to Ca<sup>2+</sup> homeostasis of multiple tissues [13-16]. When cells are in an electrically or chemically activated state, reverse-mode NCX is known to promote Na<sup>+</sup> efflux and Ca<sup>2+</sup> influx through the plasma membrane. Muscarinic agonists not only can stimulate InsP<sub>3</sub> production, but also can promote the

opening of non-selective cation channels followed by elevation of membrane potentials in various cell types [28-30]. Thus, Na<sup>+</sup> introduced through non-selective cation channels might play an important role in Ca<sup>2+</sup> influx through rNCX under muscarinic stimulation in NCI-H716 cells. In this study, KB-R7943, a rNCX blocker, effectively attenuated CCh-induced cytosolic Ca<sup>2+</sup> oscillation. Furthermore, depletion of cytosolic Na<sup>+</sup> by replacement of Na<sup>+</sup> with NMG<sup>+</sup> or Li<sup>+</sup> also slowly reduced and terminated CCh-induced cytosolic Ca<sup>2+</sup> oscillation at about 120 sec after Na<sup>+</sup> elimination. These results indicate that muscarinic agonists can activate Na<sup>+</sup>-dependent Ca<sup>2+</sup> influx which might be closely linked to the generation of oscillatory Ca<sup>2+</sup> signals in NCI-H716 cells. In addition, as a result of the experiment using cell immunostaining method, it was confirmed that the receptor of NCX1 was present on NCI-H716 cells.

Based on data presented here, it can be concluded that rNCX is very closely involved in generation of oscillatory Ca<sup>2+</sup> signals promoted by CCh in NCI-H716 cells. Thus, rNCX might play a physiologically important role as a regulator of GLP-1 secretion in NCI-H716 cells.

## FUNDING

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None.

## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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