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Short Communication

Quantitative Fluorescence Measurement of Cardiac Na^+/Ca^{2+} Exchanger Inhibition by Kinetic Analysis in Stably Transfected HEK293 Cells

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Abstract. We developed a method to quantitatively evaluate the potency of Na^+/Ca^{2+} exchanger (NCX) inhibitors with fluorescence microscopy in NCX1-transfected HEK 293 cells. The reverse mode and forward mode NCX activities were measured as the ascending slope of the early phase increase in cytoplasmic Ca^{2+} concentration after change to low Na^+ extracellular solution and the descending rate (inverse of the exponential time constant) on return to normal solution, respectively. Both modes of NCX were inhibited by SEA0400 (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline) and KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate), and the concentration-inhibition relationships for both inhibitors were in good agreement with those previously reported in voltage clamped cardiomyocytes.

Keywords: Na^+/Ca^{2+} exchanger, fluorescence microscopy, SEA0400

The Na^+-Ca^{2+} exchanger (NCX) is involved in the physiological and pathophysiological regulation of intracellular Ca^{2+} concentration in various cell types. It functions both in the forward (Ca^{2+} extrusion) and reverse (Ca^{2+} influx) modes. The major role of NCX in the myocardium is to extrude Ca^{2+} from the cell through the forward mode and maintain low cytoplasmic Ca^{2+} concentration. The involvement of reverse mode NCX has also been implicated in pathological states such as ischemia and reperfusion in the heart (1, 2), kidney (3), and brain (4); hypertension (5); and arrhythmia (6). Potent and selective NCX inhibitors would be useful for clarifying the physiological and pathophysiological roles of NCX (7).

Quantitative measurement of NCX activity has been performed with voltage clamp experiments (8 – 10) or kinetic analysis with radioisotopes (4, 11). Fluorescent measurement with ion-sensitive probes provides a simpler and safer means to detect NCX activity, but has been considered less quantitative than voltage clamp or radioisotopic measurements. In the present study, we performed kinetic analysis of the changes in cytoplasmic Ca^{2+} concentration in HEK293 cells stably expressing the cardiac type NCX and demonstrated that such analysis enables quantitative evaluation of the potency of inhibitors on both forward and reverse mode NCX.

For these studies, full-length cDNA for bovine cardiac type NCX (NCX1; GeneBank Accession No. L06438, ref. 12) was inserted into the expression vector pIRES2-EGFP (Clontech, Palo Alto, CA, USA). This vector was introduced into HEK293 cells with lipofectamine (Invitrogen, Carlsbad, CA, USA) and stable transformants were obtained by clone culture in the presence of G418, a neomycin analogue. As this vector yields a bicistronic mRNA coding both NCX and green fluorescence protein (GFP), the emission of GFP could be used as an NCX expression marker. The GFP-positive cells constantly showed NCX activity.

For the measurement of NCX activity, the cells were preincubated in normal Tyrode solution (143 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.5 mM glucose, 5 mM HEPES, and 0.33 mM NaH_2PO_4), and were loaded with 5 μM fura 2/AM for 30 min at 37°C. Ouabain (1 μM) and 3 μM cyclopiazonic acid (CPA) were present throughout the Ca^{2+} measurements, and

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10 μM monensin was present in the normal Tyrode solution only before application of low Na^+ solution. To induce Ca^{2+} influx through the reverse mode NCX, the extracellular solution was changed to a low Na^+ solution (143 mM choline Cl, 3 μM CPA, 1 μM ouabain, 1 μM atropine, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.5 mM glucose, 5 mM HEPES, and 0.33 mM NaH_2PO_4). Ca^{2+} efflux through the forward mode NCX was induced by return to normal extracellular solution. Cells on the coverslips were placed in an experimental recording chamber with approximate volume of 100 – 200 μl and continuously perfused at the rate of 10 ml/min with external solution. This method enabled the complete exchange of external solution surrounding the cells in 300 – 400 ms. The cells were excited at 340 and 380 nm from a Xenon lamp and a high-speed excitation wavelength switcher (C7733; Hamamatsu Photonics, Hamamatsu) and emission (>500 nm) was separated with a dichroic mirror and detected by a high-speed cooled CCD camera (C6790, Hamamatsu Photonics). Data acquisition and analysis were performed with Aquacosmos software (Ver. 2.5, Hamamatsu Photonics). Calibration between fluorescence ratio and Ca^{2+} concentration was performed *in situ* as previously described (13, 14).

The early phase (to about 60% peak Ca^{2+} concentration) of the increase in cytoplasmic Ca^{2+} concentration after treatment of the cells with a low Na^+ extracellular solution was fitted to a linear function by linear regression analysis. The decreasing phase of cytoplasmic Ca^{2+} concentration (between about 95% to 50% peak Ca^{2+} concentration) after return to normal Tyrode

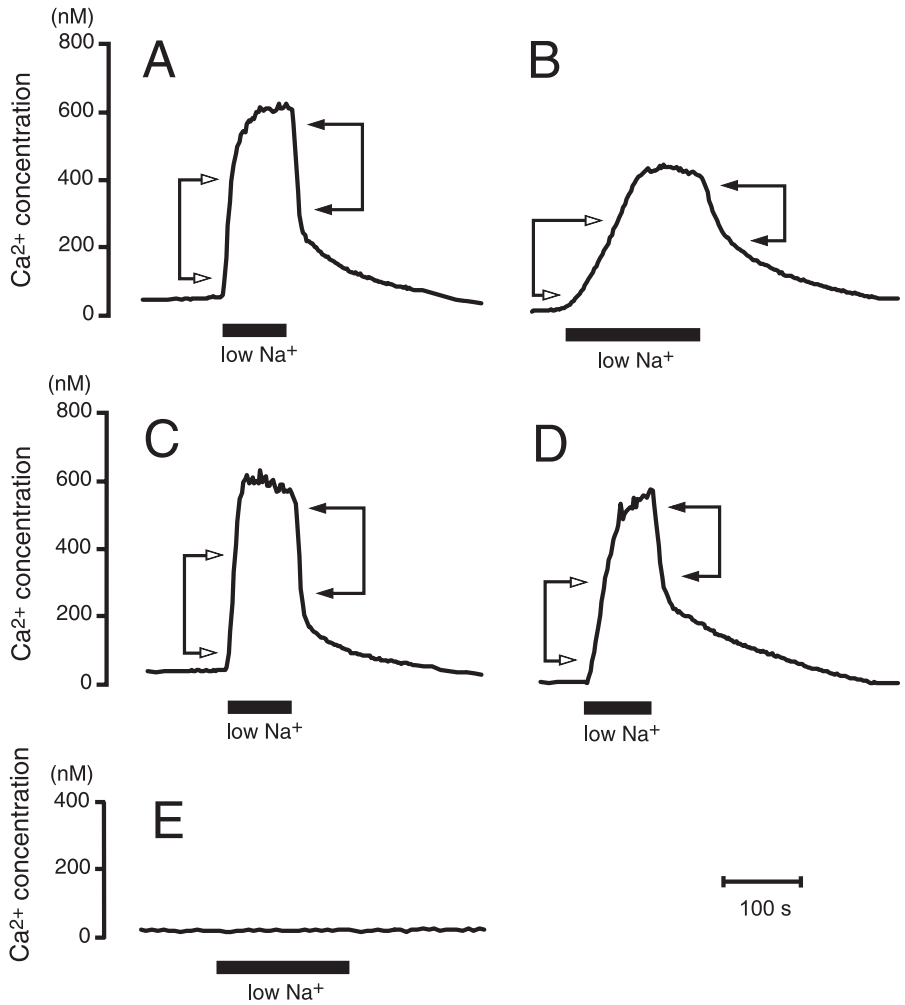

Fig. 1. Typical recording of the changes in cytoplasmic Ca^{2+} in HEK 293 cells expressing NCX 1 in the absence (A, C) and presence of SEA0400 (B, 1 μM) and KB-R7943 (D, 1 μM), respectively, and typical records from untransfected HEK293 cells (E). The reverse mode NCX was activated by changing the extracellular solution to low Na^+ solution (horizontal bars) and the forward mode NCX was activated on return to normal solution. The brackets with open and closed arrows indicate the region fit into a linear function and a single exponential decay function, respectively.

solution was fitted to a single exponential function by iterative non-linear regression.

SEA0400 (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline) was provided by Taisho Pharmaceutical Company, Ltd. (Saitama). The drug was dissolved in dimethyl sulfoxide (final concentration 0.01%). KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl] ethyl]isothiourea methanesulfonate) was kindly provided by Nihon Organon (Osaka). CPA, monensin, and ouabain were obtained from Sigma (St. Louis, MO, USA). Fura 2 was obtained from Dojin (Kumamoto). All other chemicals were of the highest commercially available quality.

Treatment of NCX-expressing HEK293 cells with a low Na^+ extracellular solution resulted in an increase in cytoplasmic Ca^{2+} concentration through the reverse mode NCX (Fig. 1). The early phase (to about 60% peak Ca^{2+} concentration) of the increase in cytoplasmic Ca^{2+} concentration showed good fit to a linear function and the slope was considered to reflect reverse mode activity. No increase in Ca^{2+} concentration was observed when untransfected HEK293 cells were treated with the low Na^+ extracellular solution (Fig. 1E).

Return to normal Tyrode solution resulted in a decrease in cytoplasmic Ca^{2+} concentration through the forward mode NCX (Fig. 1). The decreasing phase of cytoplasmic Ca^{2+} concentration (between about 95% to 50% peak Ca^{2+} concentration) showed good fit to a single exponential function, and the decay time constant was calculated. The inverse of the decay time constant was considered to be proportional to forward mode NCX activity (Fig. 1). The basal value of the ascending slope and the decay time constant were 109.3 ± 7.7 nM/s and 8.2 ± 0.5 s (n = 164), respectively.

NCX inhibitors, either SEA0400 or KB-R7943, decreased the ascending rate of Ca^{2+} concentration induced by low Na^+ solution and the declination rate of Ca^{2+} concentration induced by return to Tyrode solution (Fig. 1: B and D). The ascending slope and declination rate under a given concentration of inhibitors were normalized with the corresponding value in the absence of inhibitors. The effects of both compounds were concentration-dependent and mode-independent (forward *vs* reverse). SEA0400 was about tenfold more potent than KB-R7943 (Fig. 2, Table 1). The variation in basal ascending or descending rates did not affect the inhibition by NCX inhibitors.

The present results indicated that kinetic analyses of cytoplasmic Ca^{2+} enable quantitative measurement of drug effects on NCX activity. Fluorescent measurement with ion-sensitive probes has been considered less quantitative than voltage clamp or radioisotopic mea-

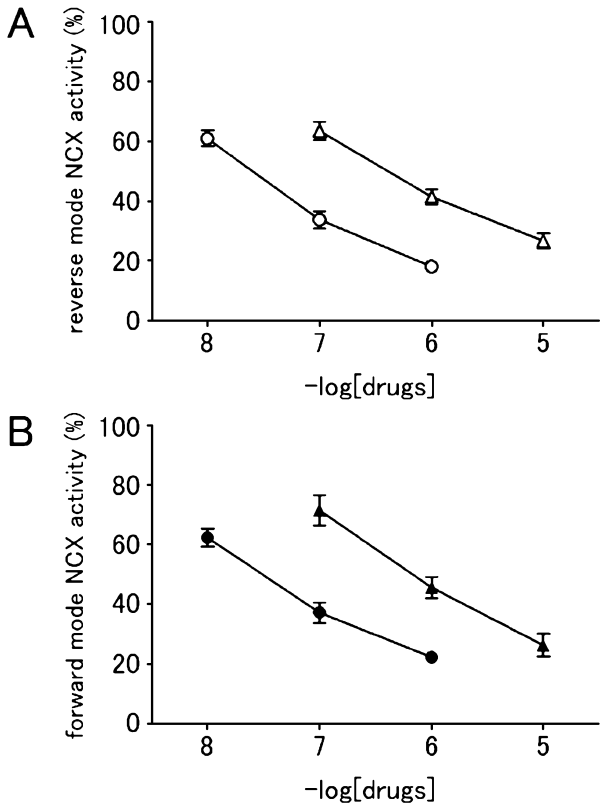

Fig. 2. Concentration-response relationships for the inhibitory effects of SEA0400 (circles) and KB-R7943 (triangles) on reverse mode (A) and forward mode (B) NCX. The ascending slope and declination rate under a given concentration of inhibitors were normalized with the corresponding value in the absence of inhibitors. Each point with vertical bars represents the mean ± S.E.M. from 20 to 30 experiments.

Table 1. EC_{50} values for inhibition of NCX

		Fluorescence microscopy	Voltage clamp
SEA0400	reverse mode	26 nM	32 nM
	forward mode	31 nM	40 nM
KB-R7943	reverse mode	419 nM	457 nM
	forward mode	682 nM	263 nM

EC_{50} values for the inhibitor of forward and reverse mode NCX obtained with fluorescence microscopy in the present study were compared with these obtained in voltage clamped ventricular myocytes (9).

surements. One of the major reasons for this lack of quantitativity was the use of steady-state fluorescence levels in analysis. Steady states levels of cytoplasmic Ca^{2+} are determined by the balance between multiple Ca^{2+} handling mechanisms and do not necessarily reflect NCX activity. Thus, in the present study, we performed kinetic analysis to extract the parameters, which theoretically reflect NCX activity. The ascending slope of the increase in cytoplasmic Ca^{2+} induced by low Na^+ solution is proportional to Ca^{2+} influx through reverse mode NCX. Fitting of the data to a linear function was performed only in the early phase where the cytoplasmic Ca^{2+} is low and the forward mode NCX was negligible. On return to normal extracellular solution, cytoplasmic Ca^{2+} is extruded by the forward mode NCX and the rate is proportional to the cytoplasmic Ca^{2+} concentration. Ca^{2+} extrusion is initially large but decreases in parallel to the cytoplasmic Ca^{2+} concentration, which theoretically results in a typical single exponential decay of cytoplasmic Ca^{2+} concentration. Fitting was performed only during the early phase of decay where cytoplasmic Ca^{2+} is high and the forward mode NCX activity is dominant. In the later phase, such relationships are obscured by the presence of other mechanisms such as reverse mode NCX and intracellular Ca^{2+} handling mechanisms.

Comparison of the present results with those obtained with voltage clamped cardiomyocytes (9, 10) proved that the present methods are valid. Inhibitory effects of the two established NCX inhibitors SEA0400 and KB-R7943 were concentration-dependent, mode-independent (forward *vs* reverse), and the potency of SEA0400 was about tenfold higher than that of KB-R7943 (Table 1). These results were in good agreement with those in voltage-clamped cardiomyocytes (9, 10); SEA0400 and KB-R7943 blocked the inward (forward mode) and outward (reverse mode) NCX currents with similar efficacy when induced by ramp pulses under bi-directional ionic conditions (9, 10). In contrast, NCX inhibitors blocked the reverse mode NCX current much more effectively than the forward mode current under unidirectional ionic conditions; SEA0400 and KB-R7943 concentration-dependently inhibited the outward current with IC_{50} values of 28 and 88 nM, respectively, but did not affect the inward currents (11). This led some investigators to postulate that these inhibitors selectively inhibit the reverse mode NCX under physiological conditions, a property desirable as therapeutic agents against cardiovascular disorders such as ischemia-reperfusion injury (1, 2) and hypertension (5). This apparent mode selectivity can be explained by the cytoplasmic Na^+ dependence of inhibition (15); thus inhibition is mode independent under simplified experimental conditions (8, 9).

Concerning the reverse mode NCX, the present results with fluorescence analyses were consistent with RI measurements under unidirectional ion conditions. Matsuda et al. reported that SEA0400 was more potent than KB-R7943 in inhibiting Na^+-dependent $^{45}Ca^{2+}$ uptake in cultured neurons, astrocytes, and microglia; and the IC_{50} values of SEA0400 and KB-R7943 were 5 to 33 nM, and 2.0 to 4 μM, respectively (4). Iwamoto reported that the IC_{50} values of SEA0400 and KB-R7943 determined by Na^+-dependent $^{45}Ca^{2+}$ uptake were 56 nM and 4.3 μM, respectively, in CCL39 cells stably expressing canine cardiac NCX1 (11). The measured inhibitory potency of KB-R7943 appeared to be more variable among reports than SEA0400; this may somehow be related to the lower selectivity of KB-R7943 for NCX (4, 9).

The kinetic analyses performed in the present study are technically simple, radioisotope-free, and, in principle, applicable to other ion transporters and channels. It may also be used in native cells provided that the activities of interfering ion transporters are inhibited. After some modification, it would be applicable to high throughput screening with multi-well plate fluorometers. Thus, kinetic analyses with fluorescent ion indicators would provide a simple and useful method for quantitative measurement of ion transporters.

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