Pharmacological Discrimination of Plasmalemmal and Mitochondrial Sodium–Calcium Exchanger in Cardiomyocyte-Derived H9c2 Cells

Iyuki Namekata,* Shogo Hamaguchi, and Hikaru Tanaka

Department of Pharmacology, Toho University Faculty of Pharmaceutical Sciences; Funabashi, Chiba 274–8510, Japan.

Received July 17, 2014; accepted October 23, 2014

Note

We examined the effects of SEA0400 and CGP-37157 on the plasmalemmal Na\(^+\)–Ca\(^{2+}\) exchanger (NCX) and mitochondrial NCX using H9c2 cardiomyocytes loaded with Ca\(^{2+}\)-sensitive fluorescent probes. The plasmalemmal NCX activity, which was measured as the increase in cytoplasmic Ca\(^{2+}\) concentration after application of low Na\(^+\) extracellular solution, was inhibited by SEA0400 but not by CGP-37157. The mitochondrial NCX activity, which was measured in permeabilized H9c2 cells as the decrease in mitochondrial Ca\(^{2+}\) concentration after application of Ca\(^{2+}\)-free extramitochondrial solution, was inhibited by CGP-37157 but not by SEA0400. These results indicate that SEA0400 and CGP-37157 act as selective inhibitors towards plasmalemmal and mitochondrial NCX, respectively, and provide pharmacological evidence that the plasmalemmal and mitochondrial NCX are distinct molecular entities.

Key words mitochondria; Na\(^+\)–Ca\(^{2+}\) exchanger; SEA0400; Ca\(^{2+}\) imaging

The Na\(^+\)–Ca\(^{2+}\) exchanger (NCX) is involved in the physiological and pathophysiological regulation of intracellular Ca\(^{2+}\) concentration in various cell types including cardiomyocytes. Although the major role of plasmalemmal NCX in cardiomyocytes is to extrude Ca\(^{2+}\) from the cell and maintain low cytoplasmic Ca\(^{2+}\) concentration, NCX is also considered to be responsible for the pathogenesis of ischemia–reperfusion and arrhythmia. Concerning pharmacological inhibition of NCX, SEA0400 [2-[4-[(2,5-difluorophenyl)methoxy]phenoxyl]-5-ethoxyaniline], a benzyloxyphenyl compound, was established as a potent inhibitor of NCX with minimum effects on other plasmalemmal ion channels and transporters. In voltage-clamped guinea pig ventricular myocytes, 1 \(\mu\)M SEA0400, which inhibited the plasmalemmal NCX current by more than 80%, had no effect on the Na\(^+\) current, L-type Ca\(^{2+}\) current, delayed rectifier K\(^+\) current, inwardly rectifying K\(^+\) channel, the Na\(^+\)–K\(^+\) channel, the Na\(^+\)–Ca\(^{2+}\) exchanger, and the Na\(^+\)–K\(^+\)–ATPase of the cardiac sarcolemma, or the Ca\(^{2+}\)–ATPase of the cardiac sarcoplasmic reticulum at the concentration of 10 \(\mu\)M. Based on these findings CGP-37157 rapidly became a privileged tool to investigate mitochondrial NCX. However, recent studies showed that CGP-37157 may also affect plasmalemmal NCX. Concerning SEA0400, whether it affects mitochondrial NCX or not has not yet been examined.

In the present study, we examined the effects of SEA0400 and CGP-37157 on the plasmalemmal and mitochondrial NCX using the rat embryonic heart derived H9c2 cardiomyocytes. We intended to examine the selectivity of these compounds towards the plasmalemmal and mitochondrial NCX, and clarify whether the plasmalemmal and mitochondrial NCX can be pharmacologically discriminated.

MATERIALS AND METHODS

Measurement of NCX Activity For the measurement of plasmalemmal NCX activity, H9c2 cardiomyocytes were preincubated in normal Tyrode solution (143 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 0.33 mM NaH\(_2\)PO\(_4\), 10 mM glucose, and 5 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES): pH 7.4 with NaOH) and were loaded with the cytoplasmic Ca\(^{2+}\) fluorescence probe indo-1 (5 \(\mu\)M indo-1/AM for 30 min at 37°C). To induce Ca\(^{2+}\) influx through the plasmalemmal NCX, the extracellular solution was changed to a low Na\(^+\) solution (143 mM choline Cl, 3 \(\mu\)M cyclopiazonic acid, 1 \(\mu\)M ouabain, 1 \(\mu\)M atrpine, 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\(_2\)PO\(_4\); pH 7.4). Ouabain (1 \(\mu\)M) and 3 \(\mu\)M cyclopiazonic acid were present throughout the Ca\(^{2+}\) measurements, and 10 \(\mu\)M monensin was present in the normal Tyrode solution before application of low Na\(^+\) solution.

Measurement of Mitochondrial NCX Activity For the measurement of mitochondrial NCX activity, H9c2 cardiomyocytes were preincubated with the mitochondrial Ca\(^{2+}\) fluorescence probe Rhod 2 (2 \(\mu\)M Rhod 2/AM for 30 min at room temperature). The plasmalemmal membrane was permeabilized by perfusion of digitonin (20 \(\mu\)g/mL) in a Ca\(^{2+}\)-free extramitochondrial solution containing (in mM) 50 KCl, 80 potassium aspartate, 4 sodium pyruvate, 20 HEPES, 3 MgCl\(_2\), 3 Na\(_2\)ATP, 5.8 glucose, and 3 ethylene glycol bis (2-aminoethyl ether)-N,N,N',N’-tetra acetic acid (EGTA) (pH 7.3 with KOH).

* To whom correspondence should be addressed. e-mail: iyuki@phar.toho-u.ac.jp
On measurement of mitochondrial NCX activity, the Ca$^{2+}$ concentration of the extramitochondrial solution was changed from 300 nM to 0 nM. The basal solution was either the same as that shown above, which contained 10 mM Na$^+$, or supplemented with NaCl up to 50 mM Na$^+$. The free Ca$^{2+}$ concentration in the extramitochondrial solution was changed according to the experimental protocol. The free Ca$^{2+}$ concentration was adjusted using the software Webmax extended.23)

**Measurement of Cytoplasmic and Mitochondrial Ca$^{2+}$**

For the measurement of cytoplasmic Ca$^{2+}$ concentration, the cells loaded with Indo-1 were excited at 350 nm and the emissions bands 395–415 nm and 470–490 nm were detected with an image separation system (W-VIEW system, Hamamatsu Photonics, Hamamatsu, Japan) and a high-speed cooled CCD camera (HISCA, Hamamatsu Photonics) at a time resolution of 500 ms. The ratio of emission intensity between short wavelength and long wavelength fluorescence was calculated (Aquacosmos software, Hamamatsu Photonics). In *situ* calibration of indo-1 fluorescence ratio values to intracellular Ca$^{2+}$ concentration was performed as previously described.24,25) For the measurement of mitochondrial Ca$^{2+}$ concentration, permeabilized cells loaded with Rhod 2 were excited 543 nm and the emission at 580–600 nm were detected at a time resolution of 10 s.

**Drugs and Chemicals**

SEA0400, kindly provided by Taisho Pharmaceutical Company, Ltd., and CGP-37157 (Tocris, Bristol, U.K.) were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the extracellular solution did not affect the parameters observed.23)

**RESULTS**

**Measurement of Plasmalemmal NCX Activity**

To clarify the effects of SEA0400 and CGP-37157 on plasmalemmal NCX, NCX activities were measured as the increase in cytoplasmic Ca$^{2+}$ concentration in intact H9c2 cells (Fig. 1). Treatment of intact H9c2 cells with a low Na$^+$ extracellular solution elicited an increase in cytoplasmic Ca$^{2+}$ concentration which reflects Ca$^{2+}$ influx through the plasmalemmal NCX. The cytoplasmic Ca$^{2+}$ concentration before and after application of low Na$^+$ solution was 127.7±14.2 and 945.3±26.9 nM, respectively (n=35). SEA0400 (1 µM) inhibited the increase in Ca$^{2+}$ concentration induced by low Na$^+$ solution (Figs. 1A, C), but CGP-37157 (10 µM) had no effect (Figs. 1B, C). The peak cytoplasmic Ca$^{2+}$ concentration

**Fig. 1. Analysis of Plasmalemnal NCX in Indo-1-Loaded H9c2 Cells**

A, B: Typical recording of the changes in cytoplasmic Ca$^{2+}$ on application of low Na$^+$ extracellular solution in intact H9c2 cells in the absence (Aa, Ba) and presence of SEA0400 (1 µM; Ab) or CGP-37157 (10 µM; Bb). C: Summarized results for the effect of inhibitors. The low Na$^+$-induced increase in cytoplasmic Ca$^{2+}$ in the presence of SEA0400 or CGP-37157 was expressed as a percentage of that in the absence. Columns and bars represent the mean±S.E.M. from 15 to 20 experiments. Asterisks indicate significant difference from corresponding values in the absence of inhibitors as evaluated by the paired *t*-test.

**Measurement of Mitochondrial NCX Activity**

To clarify the effects of SEA0400 and CGP-37157 on plasmalemmal NCX, NCX activities were measured as the increase in cytoplasmic Ca$^{2+}$ concentration in intact H9c2 cells (Fig. 1). Treatment of intact H9c2 cells with a low Na$^+$ extracellular solution elicited an increase in cytoplasmic Ca$^{2+}$ concentration which reflects Ca$^{2+}$ influx through the plasmalemmal NCX. The cytoplasmic Ca$^{2+}$ concentration before and after application of low Na$^+$ extracellular solution was 127.7±14.2 and 945.3±26.9 nM, respectively (n=35). SEA0400 (1 µM) inhibited the increase in Ca$^{2+}$ concentration induced by low Na$^+$ solution (Figs. 1A, C), but CGP-37157 (10 µM) had no effect (Figs. 1B, C). The peak cytoplasmic Ca$^{2+}$ concentration

**Fig. 2. Analysis of Mitochondrial NCX in Rhod-2-Loaded Permeabilized H9c2 Cells**

A: Typical recordings of the changes in Rhod-2 fluorescence after removal of extramitochondrial Ca$^{2+}$ (300 to 0 nM) under 10 mM Na$^+$ in the absence of inhibitors (dashed grey line) and under 50 mM Na$^+$ in the absence of inhibitors (solid grey line), in the presence of 10 µM CGP-37157 (solid black line) and in the presence of 1 µM SEA0400 (dashed black line). B: Summarized results for mitochondrial NCX activity. The decrease in fluorescence at 10 min after changing to Ca$^{2+}$-free extramitochondrial solution was expressed as a percentage of the fluorescence under 300 nM Ca$^{2+}$. Columns and bars represent the mean±S.E.M. from 10 to 20 experiments. Asterisks indicate significant difference from corresponding values in control as evaluated by the Dunnett’s test for multiple comparisons.
after application of low Na\(^+\) extracellular solution in the presence of SEA0400 and CGP-37157 was 233.1±27.7 (n=20) and 943.0±32.2 nM (n=15), respectively.

**Measurement of Mitochondrial NCX Activity** To clarify the effects of SEA0400 and CGP-37157 on mitochondrial NCX, the declining phase of mitochondrial Ca\(^{2+}\) concentration was analyzed in permeabilized H9c2 cells. The decline of mitochondrial Ca\(^{2+}\) concentration on changing the extramitochondrial Ca\(^{2+}\) concentration from 300 nM to 0 nM was dependent on extra-mitochondrial Na\(^+\) indicating that the decline reflects mitochondrial NCX activity (Fig. 2). CGP-37157 (10 \(\mu\)M) inhibited the declination of mitochondrial Ca\(^{2+}\). In contrast, SEA0400 (1 \(\mu\)M) had no effect (Fig. 2).

**DISCUSSION**

The plasmalemmal and mitochondrial NCX function either in the Ca\(^{2+}\) extrusion or Ca\(^{2+}\) influx mode, depending on the Na\(^+\) gradient.\(^{1,26}\) This enabled us to construct fluorescence-based analyzing systems of plasmalemmal and mitochondrial NCX activities and evaluate the effects of pharmacological agents. Plasmalemmal NCX activity could be elicited by exposure to low Na\(^+\) extracellular solution of H9c2 cells loaded with the cytoplasmic Ca\(^{2+}\) indicator indo-1, and the resulting increase in fluorescence reflected plasmalemmal NCX activity (Fig. 1). To detect mitochondrial NCX activity, we used permeabilized H9c2 cells loaded with the mitochondria selective Ca\(^{2+}\) indicator Rhod 2. Exposure of these cells to Ca\(^{2+}\)-free extra-mitochondrial solution resulted in a decrease in mitochondrial Ca\(^{2+}\) concentration; the decrease was dependent on Na\(^+\) indicating that the decrease indeed reflects mitochondrial NCX activity (Fig. 2). Thus, the present experimental system was shown to be suitable to study mitochondrial Ca\(^{2+}\) regulation.

Information concerning the pharmacological properties of plasmalemmal and mitochondrial NCX is limited. CGP-37157 was reported to inhibit the mitochondrial NCX without affecting the major ion channels and transporters. It did not affect the plasmalemmal NCX in isolated cardiac sarcolemmal vesicles.\(^{19}\) However, CGP-37157 was reported to block the plasmalemmal NCX (NCX 1.1) expressed in Xenopus oocytes.\(^{12,22}\) In the present study with intact cardiomyocyte-derived H9c2 cells, CGP-37157 (10 \(\mu\)M), which completely inhibited the mitochondrial NCX (Fig. 2), had no effects on plasmalemmal NCX (Fig. 1). These results indicated that CGP-37157 is a selective inhibitor of mitochondrial NCX in intact cardiac myocytes, and thus can be used as a tool for the studies of mitochondrial NCX activity. On the other hand, SEA0400 (1 \(\mu\)M), which markedly inhibited the plasmalemmal NCX (Fig. 1), had no effect on mitochondrial NCX (Fig. 2). This indicates that SEA0400 is a selective inhibitor of plasmalemmal NCX in intact cardiac myocytes, and thus can be used as a tool to obtain information on plasmalemmal NCX function.

The present results provide pharmacological evidence that the plasmalemmal and mitochondrial NCX are distinct molecular entities. The postulated ion transporting region of the plasmalemmal NCX and NCLX is formed by two transmembrane domains called the \(\alpha 1\) and \(\alpha 2\) repeats.\(^{15,27}\) NCX and NCLX shares about 62% homology within the \(\alpha 1\) and \(\alpha 2\) repeats, but not outside these regions.\(^{23}\) The discrimination by SEA0400 and CGP37157 may suggest that their binding sites on these transporters largely involve regions outside of the \(\alpha 1\) and \(\alpha 2\) repeats. This appears to be consistent with an earlier binding site model for NCX inhibitors of benzoxoxyphenyl structure such as SEA0400.\(^{15}\) In any case, mitochondrial NCX and plasmalemmal NCX could be pharmacologically discriminated, which provides a basis for the development of novel therapeutic agents.

In conclusion, we demonstrated that SEA0400 and CGP-37157 act as selective inhibitors towards plasmalemmal and mitochondrial NCX, respectively, in intact cardiomyocyte-derived H9c2 cells, which provided pharmacological evidence that the plasmalemmal and mitochondrial NCX are distinct molecular entities.

**Conflict of Interest** The authors declare no conflict of interest.

**REFERENCES**

12) Li W, Shariat-Madar Z, Powers M, Sun X, Lane RD, Garlid KD. Reconstitution, identification, purification, and immunological

13) Kar P, Chakraborti T, Samanta K, Chakraborti S. \(\mu\)-Calpain mediated cleavage of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in isolated mitochondria under A23187 induced Ca\textsuperscript{2+} stimulation. *Arch. Biochem. Biophys.*, **482**, 66–76 (2009).


