Fluorescence Discrimination of Pharmacological Effects on the Na\(^+\)-Ca\(^{2+}\) Exchanger and Sarco-Endoplasmic Reticulum Ca\(^{2+}\)-ATPase in Mouse Ventricular Cardiomyocytes

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We developed a method to evaluate the activity of the Na\(^+\)–Ca\(^{2+}\) exchanger (NCX) and sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) with fluorescence microscopy in mouse ventricular cardiomyocytes. In non-beating ventricular cardiomyocytes, \(\alpha\)-adrenoceptor stimulation by phenylephrine caused a decrease in the cytoplasmic Ca\(^{2+}\) concentration, which was inhibited by SEA0400, an NCX inhibitor, but not cyclopiazonic acid, a SERCA inhibitor. \(\beta\)-Adrenoceptor stimulation by isoprenaline caused a decrease in the cytoplasmic Ca\(^{2+}\) concentration, which was inhibited by cyclopiazonic acid but not SEA0400. Ellagic acid, a phenolic phytochemical, also decreased the basal Ca\(^{2+}\) concentration, which was inhibited by cyclopiazonic acid, but not SEA0400. Thus, this method using fluorescent microscopy and specific inhibitors would be useful for the evaluation of pharmacological agents acting on NCX and SERCA.

Key words  sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPase; Na\(^+\)–Ca\(^{2+}\) exchanger; Ca\(^{2+}\) imaging; ellagic acid

INTRODUCTION

The heart works as a pump at the center of the circulatory system by spontaneous rhythmic contraction of the myocardium. In cardiomyocytes, the action potential triggers the Ca\(^{2+}\) transient, a rapid rise in Ca\(^{2+}\) concentration in the entire cytosplasm, leading to cellular contraction. The removal of Ca\(^{2+}\) from the cytoplasm through transsarcolemmal Ca\(^{2+}\) extrusion and/or Ca\(^{2+}\) uptake into the sarcoplasmic reticulum leads to relaxation of the cardiomyocytes. Impaired myocardial relaxation is considered to play causal roles in certain types of heart failure, and drugs that improve myocardial relaxation is anticipated to be of benefit in the maintenance of the pumping heart failure, and drugs that improve myocardial relaxation is considered to play causal roles in certain types of heart failure. The removal of cytoplasmic Ca\(^{2+}\) is carried out by two major transporters, the Na\(^+\)–Ca\(^{2+}\) exchanger (NCX) on the sarcolema and the sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) on the sarcoplasmic reticulum. The activity of these transporters varies depending on the pathophysiological condition of the heart and is known to be decreased in various types of heart failure.

Cellular expression systems and sarcolemmal or sarcoplasmic reticulum membrane vesicles have been used for the evaluation of pharmacological effects on NCX and SERCA. Analyses with these systems, however, require the use of radioisotopes or voltage clamp techniques. In some cases, results obtained in such systems do not accurately predict the pharmacological profile in cardiomyocytes.

In the present study, we intended to develop a simple fluorescence-based method to evaluate pharmacological effects of agents on NCX and SERCA in intact cardiomyocytes. In beating cardiomyocytes, however, Ca\(^{2+}\) supplying mechanisms such as the L-type Ca\(^{2+}\) channel and the ryanodine receptor Ca\(^{2+}\) release channel as well as Ca\(^{2+}\) removal mechanisms such as NCX and SERCA are activated altogether during the contraction-relaxation cycle, which makes it difficult to evaluate the contribution of each transporter. To avoid this problem, we used non-beating cardiomyocytes whose L-type Ca\(^{2+}\) channel and ryanodine receptor activities were minimum. The basal cytoplasmic Ca\(^{2+}\) concentration was measured with fluorescence microscopy, and NCX and SERCA were activated through \(\alpha\)-adrenergic stimulation and \(\beta\)-adrenergic stimulation, respectively. The involvement of NCX and SERCA was confirmed with SEA0400 and cyclopiazonic acid, selective inhibitors of NCX and SERCA, respectively. As the results appeared to be successful, we applied the method for the evaluation of ellagic acid, a phenolic phytochemical, to further confirm the validity of this method in the evaluation of novel compounds.

MATERIALS AND METHODS

All experiments were performed in accordance to the “Guiding Principles for the Care and Use of Laboratory Animals” approved by The Japanese Pharmacological Society. Ventricular myocytes were prepared from adult mice by Langendorff perfusion and collagenase digestion as in our previous study. For the measurement of intracellular Ca\(^{2+}\) concentration, cells were preincubated for 30 min with 5 \(\mu\)M Fura-2-AM, superfused with Tyrode’s solution of the following composition (mM): NaCl 143, KCl 4, MgCl\(_2\) 0.5, CaCl\(_2\) 1.8, NaH\(_2\)PO\(_4\) 0.33, glucose 5.5 and N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) 5, and observed with a fluorescence microscope (IX70, Olympus, Tokyo, Japan). The Ca\(^{2+}\) fluorescence of single myocytes was measured and the time course on application of agents was
analyzed. The cells were excited at 340 and 380nm from a Xenon lamp and the emission (>500nm) was separated with a dichroic mirror, detected by a cooled CCD camera (C6790, Hamamatsu Photonics, Shizuoka, Japan) at a time resolution of 5s, and ratioed after correction of background fluorescence (Aquacosmos software, Hamamatsu Photonics). In *in situ* calibration of Fura-2 fluorescence ratio values to intracellular Ca\(^{2+}\) concentration was performed as previously described.\(^9\) All data were expressed as the mean ± standard error of the mean (S.E.M.). Data were analyzed by one-way ANOVA followed by Dunnett’s multiple test or the paired t-test. A p value less than 0.05 was considered statistically significant.

RESULTS

The isolated cardiomyocytes used were rod shaped and had cross striations at regular intervals (Fig. 1Aa). When loaded with the Ca\(^{2+}\) indicator, Fura-2, they showed an even fluorescence throughout the cytoplasm (Fig. 1Ab). The nuclei could be distinguished from the cytoplasm by their higher fluorescence intensity. The cellular fluorescence completely disappeared after permeabilization of the sarcolemma with digitonin (Fig. 1Ad), indicating the presence of the fluorescent indicator in the cytoplasmic component.

The mean basal cytoplasmic Ca\(^{2+}\) concentration within the cytoplasm was 101.1 ± 0.4 nM (n = 50). SEA0400 (1 \(\mu\)M), an inhibitor of NCX,\(^7\) had no effect on the Ca\(^{2+}\) concentration; the cytoplasmic Ca\(^{2+}\) concentration in the presence of 1 \(\mu\)M SEA0400 was 100.5 ± 0.5 nM (n = 30). Cyclopiazonic acid (10 \(\mu\)M), an inhibitor of SERCA,\(^8\) slightly, but significantly, increased the Ca\(^{2+}\) concentration; the cytoplasmic Ca\(^{2+}\) concentration in the presence of 10 \(\mu\)M cyclopiazonic acid was 106.6 ± 1.0 nM (n = 30).

Phenylephrine, at concentrations of 10 and 100 \(\mu\)M, decreased the cytoplasmic Ca\(^{2+}\) concentration; the maximum decrease was reached in about 5 min. The magnitude of the decrease was dependent on the concentration of phenylephrine (Fig. 1B). The decrease in Ca\(^{2+}\) concentration induced by 100 \(\mu\)M phenylephrine was completely inhibited by 1 \(\mu\)M prazosin (Figs. 1B, D), which confirms the involvement of \(\alpha\)-adrenergic receptors. The phenylephrine-induced decrease in Ca\(^{2+}\) concentration was inhibited by 1 \(\mu\)M SEA0400, but not by 10 \(\mu\)M cyclopiazonic acid (Figs. 1C, D), indicating the involvement of NCX but not SERCA.

Isoprenaline, at concentrations of 10 and 100 \(\mu\)M, decreased the cytoplasmic Ca\(^{2+}\) concentration; the maximum decrease was reached in about 5 min. The magnitude of the decrease was dependent on the concentration of isoprenaline (Figs. 2A, C). The decrease in Ca\(^{2+}\) concentration induced by 100 \(\mu\)M isoprenaline was significantly inhibited by 1 \(\mu\)M propranolol (Figs. 2A, C), which confirms that the response was mediated by \(\beta\)-adrenoceptors. The isoprenaline-induced decrease in Ca\(^{2+}\) concentration was completely inhibited by 10 \(\mu\)M cyclopiazonic acid, but not by 1 \(\mu\)M SEA0400 (Figs. 2B, C), indicating the involvement of SERCA but not NCX.

Ellagic acid (10 \(\mu\)M) induced a decrease in cytoplasmic Ca\(^{2+}\) concentration (Fig. 3A); the maximum decrease was reached in about 5 min. The magnitude of the decrease was about the same as that by 10 nM isoprenaline. The ellagic acid-induced decrease in Ca\(^{2+}\) concentration was affected neither by 1 \(\mu\)M prazosin nor by 1 \(\mu\)M propranolol (Figs. 3A, C).
It was inhibited by 10 µM cyclopiazonic acid, but not by 1 µM SEA0400 (Figs. 3B, C), indicating the involvement of SERCA but not NCX.

**DISCUSSION**

The present study was undertaken to develop a fluorescence-based method to evaluate the activity of NCX and SERCA in intact non-beating cardiomyocytes. Under non-beating conditions, a small amount of Ca\(^{2+}\) leakage from the sarcoplasmic reticulum occurs which is observed as spontaneous Ca\(^{2+}\) sparks.\(^{10}\) This Ca\(^{2+}\) is sequestered into the sarcoplasmic reticulum by SERCA, and a basal cytoplasmic Ca\(^{2+}\) concentration of about 100 nM is maintained. The present observation that cyclopiazonic acid slightly increased cytoplasmic Ca\(^{2+}\) concentration is consistent with such view.

Theoretically, interventions which enhance the activity of NCX or SERCA would change the balance between Ca\(^{2+}\) supply and removal, and result in a decrease in cytoplasmic Ca\(^{2+}\) concentration.

We previously reported that α-adrenergic stimulation resulted in enhancement of the NCX current in voltage-clamped adult mouse ventricular cardiomyocytes and a decrease in contractile force in isolated ventricular tissue preparations.\(^{6}\) These results suggested that α-adrenergic stimulation enhances Ca\(^{2+}\) efflux through NCX. The present results that phenylephrine caused a decrease in the basal Ca\(^{2+}\) concentration of non-beating cardiomyocytes indicated that enhancement of forward mode NCX (Ca\(^{2+}\) extrusion) can be observed as a decrease in basal Ca\(^{2+}\) concentration. This was further confirmed by inhibition of the decrease by SEA0400, which blocks the forward and reverse (Ca\(^{2+}\) influx) modes of NCX with minimum effects on the L-type Ca\(^{2+}\) current.\(^{7}\) The decrease was not affected by cyclopiazonic acid indicating that SERCA was not involved. Thus, this method provides a means to evaluate drug effects on NCX activity.
β-Adrenergic stimulation not only increases the contractile force, but also enhances myocardial relaxation. The latter has also been attributed to a decrease in Ca²⁺ sensitivity of the contractile protein and the enhancement of Ca²⁺ uptake by SERCA. The present results that isoprenaline caused a decrease in the cytoplasmic Ca²⁺ concentration in non-beating cardiomyocytes indicated that stimulation of SERCA can be detected by a decrease in basal Ca²⁺ concentration. This was further confirmed by inhibition of the decrease by cyclopiazonic acid, which blocks SERCA activity with minimum effects on the L-type Ca²⁺ current and Ca²⁺ release from the sarcoplasmic reticulum. SEA0400 had no effect indicating that NCX was not involved in this decrease. Thus, this method provides a means to evaluate drug effects on SERCA activity.

Ellagic acid is a phenolic chemical contained in fruits and vegetables which accelerates myocardial relaxation without affecting the contractile force. This acceleration was reduced by cyclopiazonic acid suggesting the involvement of SERCA. Activation of SERCA by ellagic acid was reported in sarcoplasmic reticulum vesicles from canine myocardium. It was postulated that ellagic acid interacts with SERCA and dissociates it from the inhibitory action of phospholamban, leading to an apparent stimulation of the enzymatic activity. However, whether ellagic acid affects the NCX has not yet been reported. In the present study, ellagic acid decreased the cytoplasmic Ca²⁺ concentration, which was inhibited by cyclopiazonic acid, but not by SEA0400 (Fig. 3). Thus, the ellagic acid-induced decrease in cytoplasmic Ca²⁺ concentration could be explained by activation of SERCA but not NCX. We reported that ellagic acid ameliorates the impairment of myocardial relaxation accompanying diabetes mellitus in the mouse. At present, a specific activator of SERCA is not clinically available. Istaroxime, a compound with both activating effect on SERCA and inhibitory effect on Na⁺-K⁺ ATPase, appears to be a promising therapeutic agent against heart failure. The overall pharmacological property and clinical potential of ellagic acid and related compounds remain to be clarified.

In the present study, the activities of NCX and SERCA could be observed as a decrease in the basal cytoplasmic Ca²⁺ concentration in the intact cardiomyocyte. Use of non-beating cardiomyocytes and selective pharmacological agents enabled clear discrimination of NCX and SERCA activities. In contrast to Ca²⁺ transient assays with beating cardiomyocytes, which require fluorescence measurements with millisecond time resolution, this assay can be easily performed with standard type fluorescence microscopes. Thus, the presently established “basal Ca²⁺ analysis method” would be useful for the evaluation of pharmacological agents acting on NCX and SERCA.

Conflict of Interest The authors declare no conflict of interest.

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