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The endogenous inhibitor of NCX1 does not resemble the properties of digitalis compound☆

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Abstract

In our previous study, we ware successful in isolation and purification of an endogenous inhibitor of the Na/Ca exchanger (NCX1) from the calf ventricle extracts. The purified factor has characterized to have strong positive inotropic effect on isometric contractions of isolated ventricle strips of guinea pig. A possibility is that besides the NCX1 the endogenous factor may also interact with other ion-transport systems (e.g., Na,K-ATPase) involved in modulation of muscle contractility-relaxation. Therefore, a primary goal of the present study was to detect a possible effect of newly found NCX1 inhibitor on Na,K-ATPase and Ca-ATPase activities. The preparations of isolated sarcolemma vesicles were used for this goal. Although the crude extracts of calf ventricles can inhibit both the Na/Ca exchange and Na,K-ATPase, these two inhibitory activities can be separated on the Sephadex G-10 column, meaning that different molecular entities might be responsible for inhibition of Na/Ca exchange and Na,K-ATPase. Addition of 100 U of purified endogenous factor to the assay medium results in nearly complete inhibition of forward (Na_i-dependent Ca-uptake) and reverse (Na_o-dependent Ca-efflux) modes of Na/Ca exchange. On the other hand, no effect was detected on activities of Na,K-ATPase and Ca-ATPase even in the presence of 500 U of purified factor in the assay medium. In light of the present data, it is concluded that the endogenous inhibitor of NCX1 does not resemble the targeting properties of digitalis like compound. Obviously, more systematic studies are required in the future for resolving a possible interaction of the endogenous inhibitor of NCX1 with other ion-transport systems involved in calcium homeostasis and action potential.

Keywords: Na–Ca exchange; Endogenous inhibitor; Inotropic factor; EC-coupling; Calcium; Endogenous digitalis; Contractile force

The cell membrane Na/Ca exchanger (NCX1) is ubiquitously distributed ion-transport system, which plays an important role in regulating the intracellular calcium homeostasis in many cell types [1]. This system is especially important for tuning a dynamic balance of cytosolic calcium in myocytes during the action potential [2–5]. Typically, NCX1 functions in a forward (Ca-extrusion) mode but under physiologically altered conditions (e.g., $\uparrow[Na]_i$, $\downarrow[Ca]_i$ and the lengthening of the action potential in heart failure) the reverse (Ca-entry) mode may increase dramatically [4–8]. Moreover, NCX1 becomes overexpressed in heart failure or hypertrophy, thereby reducing the SR Ca-load [6–8]. Although the overexpression of NCX1 may represent an adaptive response to avoid the SR Ca-overload at initial stages of cardiac disease, this may lead to contractile dysfunction (SR Ca-depletion) and arrhythmia [7,8].

It is believed that the inhibition of overexpressed NCX1 could be beneficial for suppression of arrhythmia and enhancement of muscle contractility, although some potential shortcomings must be taken into account [9,10]. A number of NCX1 blockers have been synthesized and tested in the past, but none of them are highly selective to NCX1 [2,5,9,10,27]. Besides the selectivity problem, most organic blockers inhibit preferentially

☆ *Abbreviations:* Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BAPTA, 1,2-bis(2-aminophenoxyl)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; fluo-3, *N*-[2-[2[bis(carboxymethyl)amino]-5-(2,7dichloro-6-hydroxy-3-oxy-3*H*-xanthen-9-yl)phenoxy]ethoxy]-4 methylphenyl]-*N*-(carboxymethyl)glycine; TCA, trichloroacetic acid; FRCRCFa, cyclic hexapeptide Phe–Arg–Cys–Arg–Cys–Phe-NH_2 with intramolecular S–S bond.

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either the forward or reverse mode of NCX1 which could be associated with specific problems [9,10]. For example, the inhibition of forward mode can directly prohibit the arrhythmogenic current and increase the inotropic performance, but this may be associated in potential with SR Ca-overload and diastolic dysfunction [7–11]. On the other hand, the inhibition of reverse mode could be beneficial for preventing the Ca-overload, but this may lead to unwanted negative inotropy. We developed a synthetic cyclohexapeptide (FRCRCFa) blocker of NCX1, which inhibits the inward and reverse modes with high potency and selectivity [12–15]. Moreover, FRCRCFa can inhibit the arrhythmogenic transient inward current in myocytes of hypertrophied hearts [16], but unfortunately the usage of this blocker is inadequate in most physiological experiments because of low cell permeability [17].

Recently, we hypothesized that the cardiac tissue contains a putative endogenous inhibitor of NCX1, which may have a potency to counterbalance the overexpression of NCX1 under physiologically related conditions. In agreement with this proposal, we found that the extracts of calf cardiac ventricles contain a low molecular weight endogenous factor that can inhibit both the Na/Ca exchange and its partial reaction, the Ca/Ca exchange [18]. The inhibitory activity has been purified ($\sim 10^5$-fold) by using the advanced techniques of normal and reverse phase HPLC [18]. The FAB (Fast Atomic Bombarding) mass-spectral analyses of extensively purified preparations have shown the m/z signals in the range of 350–550 Da [18]. More advanced mass-spectra and NMR techniques are currently underway for structural resolution of active compound in question. Rapid kinetic experiments have shown that the mixing of isolated endogenous factor with sarcolemma vesicles results in inhibition of NCX1 within few milliseconds, meaning that the inhibitory ligand may interact with the NCX1 protein or in its vicinity [18]. The purified endogenous inhibitor of NCX1 can enhance 6–7-fold the isometric contractions of guinea pig ventricle strips in dose–response and reversible manner, while showing no signs of digitalis like poisoning [18].

Since the characterization of the endogenous inhibitor of NCX1 has just started, it seems reasonable to test the effects of active compound on other ion-transport systems involved in Ca-homeostasis and EC-coupling. As an entry project for selectivity tests, we examine here a capacity of the endogenous inhibitor of NCX1 to affect the activity of sarcolemmal Na,K-ATPase and Ca-ATPase. These selectivity tests are justified because in potential, the inhibition of primary ion-pumps may contribute to inotropic modulation of muscle contractility.

The present studies demonstrate that the endogenous inhibitor of NCX1 does not affect the activity of the Na,K-ATPase or Ca-ATPase, even under conditions in which the added doses are well above the levels yielding a complete inhibition of NCX1. Therefore, it is concluded that the endogenous inhibitor of NCX1 does not resemble the properties of endogenous digitalis like compound.

Materials and methods

Materials and general procedures. Protease inhibitors (PMSF, pepstatin, leupeptin, and aprotenin), deoxyribonuclease I (type DN-25, obtained from bovine pancreas), and EGTA were obtained from Sigma (St. Louis, MO). Sephadex G-10 (fine) was from Pharmacia (Sweden, Upsala). The glass microfiber filters (GF/C Whatman) were from Tamar (Jerusalem, Israel). $^{45}CaCl_2$ (10–30 mCi/mg) was purchased from DuPont NEN (Boston, MA). Fluo-3 was from Teflabs (Austin, TX). The scintillation cocktail Opti-Fluor for radioactivity counting was from Packard (Groningen, Netherlands). All other reagents used were of analytical grade (>99.9%). The solutions were prepared with deionized water (18 MΩ/cm).

Preparation of sarcolemma vesicles and assays. Sarcolemma vesicles were obtained from fresh calf hearts by using the buffers containing DNase and protease inhibitor cocktail (PMSF, pepstatin, leupeptin, and aprotenin) [12,19,20]. The sarcolemma vesicles were stored in Mops/Tris, pH 7.4, and 0.25 M sucrose at −70 °C (no loss of Na/Ca exchange activity has been detected for eight months at least). Under standard assay conditions (see below), a typical activity of Na/Ca exchange was 1–2 nmol Ca/mg/s. The reaction of Na_i-dependent ^{45}Ca-uptake (37 °C) was initiated by rapid dilution of Na-loaded (160 mM) sarcolemma vesicles (20–50 μg protein) into the assay medium (0.2–0.5 ml) containing 20 mM Mops/Tris, pH 7.4, 0.25 M sucrose, and 35 μM $^{45}CaCl_2$ ($1–3 \times 10^6$ cpm/nmol) [18–21]. The reaction of Na_i-dependent ^{45}Ca-uptake was stopped by rapid injection of EGTA containing buffer into the reaction mixture [20–22]. Duration of the ^{45}Ca-uptake was controlled by electrically triggered timer devise [19–23]. The quenched reaction mixtures were filtered on the GF/C filters, washed with the EGTA buffer, and the intravesicular ^{45}Ca-contents were counted with scintillation cocktail (Opti-Fluor, Packard BioScience) for aqueous solutions [12,20–23].

The reverse mode of Na/Ca exchange (Na_o-dependent Ca-efflux) was detected (37 °C) by monitoring the fluorescence probe fluo-3 in the stopped-flow machine (SFM-3, BioLogic, France), equipped with the TC-100/15 cuvette (40 μl volume/10 mm light path) and R-376 photomultiplier (Hamamatsu, Japan). In stopped-flow experiments, the Ca-loaded vesicles (0.25 mM) were mixed with Na/BAPTA/fluo-3 buffer and the Ca-efflux was monitored by extravesicular fluo-3 (3 μM). Typically, 50 μl of Ca-loaded vesicles in MTS buffer (20 mM Mops/Tris, pH 7.4, and 0.25 M sucrose) were mixed with 50 μl Na/BAPTA/fluo-3 buffer (20 mM Mops/Tris, pH 7.4, 320 mM NaCl, 0.6 mM BAPTA, and 6 μM fluo-3). Optimal conditions for mixing (flow rate, volume, etc.) were computed by the MPF program. The data analysis was carried out by the BioKin 0.14 program equipped with the 'Pad-Laplace' and 'Simplex' modes (BioLogic, France). Nonspecific signals were recorded and subtracted as described previously [18,24]. All other details for stopped-flow experiments are described elsewhere [18,19,24].

The Na,K-ATPase and Ca-ATPase activities were measured (37 °C) by using a colorimetric assay of released inorganic phosphate [25]. The sarcolemma vesicles (2–3 mg protein/ml) were treated with 0.1% SDS and diluted 20-fold into the assay medium (0.2–0.9 ml) to give 40–60 μg protein. The reaction of ATPase activity was quenched by addition of TCA (40%) to give a final concentration of 15%. The assay medium for Na,K-ATPase consists of 50 mM Mops/Tris, pH 7.4, 4 mM $MgCl_2$, 1 mM ATP, 100 mM NaCl, 10 mM KCl, and 0.9 mM EGTA. The assay medium for Ca-ATPase contained 50 mM Mops/Tris, pH 7.4, 4 mM $MgCl_2$, 1 mM ATP, and 20 μM $CaCl_2$. In blanks, the TCA was added first and then the sarcolemma vesicles.

Extraction and purification of NCX1 inhibitory activity. The calf ventricle extracts were obtained as described previously [18]. Briefly, the frozen ventricle tissue (4–8 kg) was lyophilized, powdered, and then extracted 4–7 times by 90–95% ethanol (2 L solvent/kg lyophilized tissue). After evaporation of ethanol, the suspension was centrifuged and the supernatant was extracted several times by chloroform to remove lipids. The aqueous phase was lyophilized, dissolved in 20–30 ml distilled water, and centrifuged to remove all insoluble materials. The supernatant ($\sim 1–2 \times 10^6$ inhibitory units in total) was applied on the Sephadex G-10 column (5.5×70 cm) and active fractions were collected. The lyophilized fractions were dissolved in minimal volume of water and loaded on the preparative reverse phase column, YMC-Pack C_{30} (YMC, Japan, 20×250 mm, 16 μm particle size). The active fractions from 20 to 30 runs were collected, lyophilized, dissolved in 70% acetonitrile (or isopropanol), and applied on the normal phase preparative column, TSK-gel Amide-80 (TosoHaas, Japan) [18]. Finally, the active fractions were purified on the Cadenza CD-C18 column (3×250 mm, 3 μm particle size, Imtact, Japan), following the GS-220 HQ column (7.6×300 mm, 6 μm particle size, Shodex Asahipak, Japan). The final yield of single batch preparation was in the range of 20,000–100,000 inhibitory units (~2–5% of crude ethanol/water extract). Purified preparations of NCX1 inhibitor were lyophilized, dissolved in minimal volume of deionized water, and stored at −70 °C (no loss in inhibitory activity was found for six months at least). For standardization of inhibitory capacity, each batch of purified preparation was carefully titrated for inhibition of Na/Ca exchange (for details see above the Na_i-dependent ^{45}Ca-uptake assay). By definition one inhibitory unit represents 1% inhibition of Na_i-dependent ^{45}Ca-uptake [18].

Isometric contractions of muscle strips. Ventricle strips from guinea pig were placed in Krebs–Henseleit solution, equilibrated with O_2/CO_2 (95%/5%). For measuring the contractile force isometrically, about 5 mm length strips were placed between two platinum-wire electrodes (2 mm apart) in a Lucite holder attached to a force transducer (Grass FT03C) by surgical thread [18]. Muscle strips were immersed in 10 ml organ bath (37 °C) and continuously gassed with O_2/CO_2 (95%/5%). Isometric contractions were initiated by applying the external stimuli at 1.0–1.5 Hz. Care has been taken to ensure that the applied experimental conditions are appropriate for supra-maximal stimulation. Usually, isometric twitches were evoked with stimulation voltage ~20% above threshold at a length at which maximum steady-state twitch force was reached. Each batch of purified preparations was tested for their effects on isometric contractions. Typically, a dose of 20–30 U/ml in the organ bath enhances 6–7-fold the isometric contractions of ventricle strips obtained from guinea pig hearts.

Results and discussion

The effects of NCX1 inhibitor were tested on Na,K-ATPase and Ca-ATPase activities by using well-established assay procedures in isolated sarcolemma vesicles. The used experimental setup is very useful for proposed selectivity studies because all activities of interest (Na,K-ATPase, Ca-ATPase, Na_i-dependent Ca-uptake, and Na_o-dependent Ca-efflux) can be conveniently measured in the same preparation of vesicles. Another advantage is that in this membrane preparation most vesicles have the inside-out orientation, so bath solutions are exposed to the cytosolic side [12,19–23]. In all experiments, the NCX1 inhibitor was added to the assay medium (10–20 min prior to experiment) and the enzymatic reactions were initiated by rapid dilution of sarcolemma vesicles into the assay medium (see Materials and methods).

At initial stages of investigation, the crude extracts (lipid and protein free) were tested for their effects on Na/K ATPase (Fig. 1A) and Na/Ca exchange (Fig. 1B). In these experiments, various amounts of lyophilized crude extracts were dissolved in water and tested for their effects on Na_i-dependent ^{45}Ca-uptake or Na,K-ATPase activity (for details see Materials and methods). The increasing doses of crude extract inhibit both the Na_i-dependent ^{45}Ca-uptake and Na,K-ATPase activities although 10–20 times higher volumes of extract are required for equipotent inhibition of Na,K-ATPase activity (Fig. 1). These data indicate two possibilities:

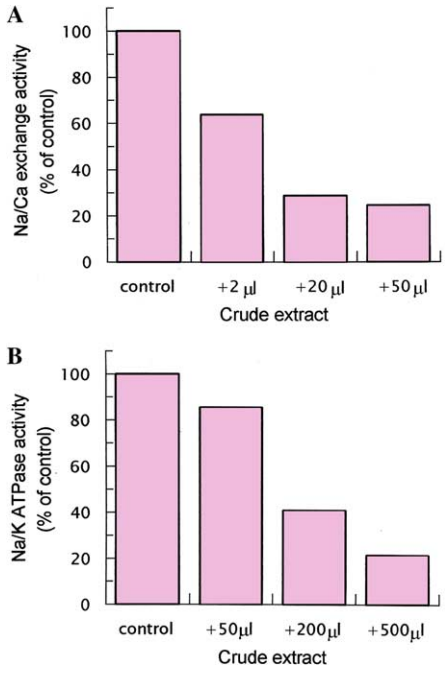

Fig. 1. Effect of ventricle crude extracts on Na/Ca exchange and Na,K-ATPase activity. Lipid and protein free extracts were tested for their effects on forward mode Na/Ca exchange (A) and Na,K-ATPase activity (B). (A) As much as 2, 20, and 50 μl of crude extracts were lyophilized, dissolved in deionized water, and added to the assay medium of Na_i-dependent ^{45}Ca-uptake. The reaction was started by rapid dilution of Na-loaded vesicles (160 mM NaCl) into the assay medium. The ^{45}Ca-uptake was stopped after 20 s by electrically controlled injection of EGTA containing buffer into the reaction mixture. The ^{45}Ca-contents in sarcolemma vesicles were assayed by filtration as described in Materials and methods. The control (100%) represents the Na_i-dependent ^{45}Ca-uptake (20 nmol ^{45}Ca/mg protein, $t = 20$ s) in the absence of extract in the assay medium. (B) As much as 50, 200, and 500 μl of crude extracts were lyophilized, dissolved in deionized water, and added to the assay medium of Na,K-ATPase. The enzymatic reaction was initiated by rapid dilution of sarcolemma vesicles into the assay medium (see Materials and methods). The ATPase activity was arrested after 5 min of reaction initiation by adding the TCA to give a final concentration of 15%. The released inorganic phosphate was assayed by colorimetric method as described in Materials and methods. The control value (100% activity) of Na,K-ATPase was 4 μmol P_i/mg protein under given experimental conditions.

either the same substance inhibits both the Na/Ca exchanger and Na,K-ATPase with different affinity or alternatively, the distinct substances are responsible for inhibiting the exchanger and Na-pump activities. In order to distinguish between these two possibilities, an attempt has been made to separate the inhibitory activities by applying the crude extracts on the Sephadex G-10 column. A clue for this experimental approach was suggested by our previous finding indicating that the molecular weight of putative endogenous inhibitor of NCX1 is in the range of 300–550 Da [18]. Fig. 2 shows that the inhibitory activities of Na/Ca exchange and Na,K-ATPase can be separated by gel-filtration on the Sephadex G-10 column. In general, the existence of Na,K-ATPase inhibitory factor in crude extracts of calf cardiac tissue is not surprising, because a number of digitalis like substances have been found in various tissue extracts [26]. The important point here is that the inhibitory activities of Na,K-ATPase and Na/Ca exchange can be effectively separated by size exclusion chromatography (gel-filtration), suggesting that the NCX1 and Na,K-ATPase inhibitors may have distinct molecular weights (Fig. 2). Moreover, we demonstrated before that the endogenous NCX1 inhibitor has a very low retention time on the reverse phase HPLC columns, meaning that it does not resemble the properties of digitalis like compound containing a steroid moiety [18]. All these data support the idea that crude extracts contain distinct molecular entities inhibiting the Na/Ca exchanger and Na-pump.

Despite the fact that the Sephadex G-10 column can separate the inhibitory activities of Na/Ca exchange and Na,K ATPase, a possibility remains that the endogenous inhibitor of NCX1 may have a low potency for inhibition of Na,K-ATPase. For testing this possibility, the experiments were designed for estimating the quantitative limits for inhibitory selectivity. In these experiments, the fixed doses of purified preparations of endogenous inhibitor of NCX1 (see Materials and methods) were added to the assay medium and Na/Ca exchange (either the forward or reverse mode), Na,K-ATPase, and Ca-ATPase activities were monitored. The time-course of Na_i-dependent ^{45}Ca-uptake and Na_o-dependent Ca-efflux (assayed by fluo-3 fluorescence probe in the stopped-flow machine) was measured in the absence or presence of 100 U of NCX1 inhibitor. This dose inhibits either the Na_i-dependent ^{45}Ca-uptake (Fig. 3B) or Na_o-dependent Ca-efflux (Fig. 4B) by

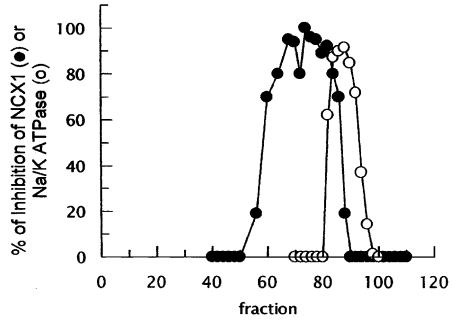

Fig. 2. Separation of inhibitory factors of Na,K-ATPase and Na/Ca exchange by gel-filtration. Lyophilized extracts (lipid and protein free) were dissolved in deionized water and applied on the preparative Sephadex G-10 column (5.5 × 70 cm). The fractions of 10 ml were collected at a flow rate of 2 ml/min (25 °C). Aliquots (100 μl) were taken from each fraction, lyophilized, and dissolved in deionized water. These samples were tested for their effects on Na_i-dependent ^{45}Ca-uptake (○) and Na,K-ATPase activity (●) according to the procedures described in Materials and methods. The inhibitory potency is expressed as percentage of inhibition (100% represents a complete inhibition of either Na/Ca exchange or Na,K-ATPase activity under standard assay conditions).

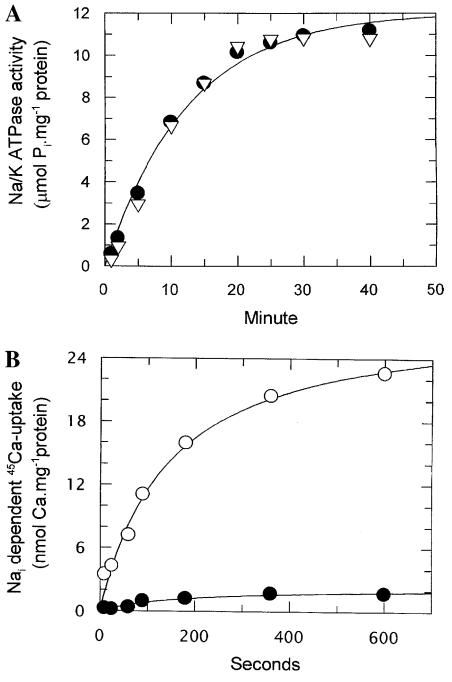

Fig. 3. Comparing the effects of isolated NCX1 inhibitor on Na,K-ATPase and Na_i-dependent ^{45}Ca-uptake. Purified preparations of NCX1 inhibitor have been tested for their effects on Na/K ATPase (A) and Na_i-dependent ^{45}Ca-uptake (B). (A) The time-course of Na/K ATPase activity was monitored in the absence (●) or presence (▽) of 500 U of NCX1 inhibitor. Ten to twenty minutes before the experiment, the NCX1 inhibitor was diluted (10-fold) into the Na,K-ATPase assay medium and the reaction of ATPase activity was initiated by rapid dilution of sarcolemma vesicles into the assay medium (see Materials and methods). At indicated times, the ATPase activity was arrested by addition of concentrated TCA and inorganic phosphate measured by colorimetric assay (see Materials and methods). (B) The time course of Na_i-dependent ^{45}Ca-uptake was measured in the absence (○) and presence (●) of 100 U of NCX1 inhibitor in the assay medium. Before the experiment the NCX1 inhibitor was diluted (50-fold) in the standard assay medium (see Materials and methods). The Na_i-dependent ^{45}Ca-uptake was initiated by rapid dilution of Na-loaded (160 mM) sarcolemma vesicles in the assay medium. At indicated times the ^{45}Ca-uptake reaction was stopped by rapid injection of EGTA containing buffer in the assay medium and the intravesicular ^{45}Ca was measured by filtration of quenched solutions on the GF/C filters (see Materials and methods).

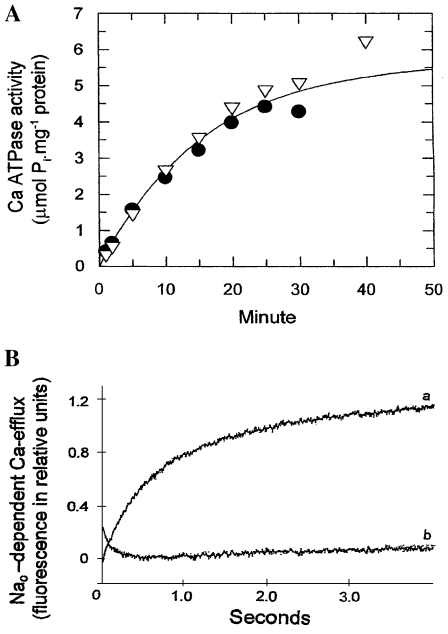

Fig. 4. Comparing the effects of isolated NCX1 inhibitor on SL Ca-ATPase and Na_o-dependent Ca-efflux. Purified preparations of NCX1 inhibitor have been tested for their effects on SL Ca-ATPase (A) and Na_o-dependent Ca-efflux (B). (A) The time-course of SL Ca-ATPase activity was monitored in the absence (●) or presence (▽) of 500 U of NCX1 inhibitor in the assay medium. Before the experiment the NCX1 inhibitor was diluted into the Ca-ATPase assay medium. The reaction of ATPase activity was started by rapid dilution of sarcolemma vesicles into the assay medium and at indicated times the phosphate release was quenched by adding the concentrated TCA (see Materials and methods). Inorganic phosphate levels were assayed by colorimetric method of Ames as described in Materials and Methods. (B) The stopped-flow kinetics of reverse mode Na/Ca exchange (Na_o-dependent Ca-efflux) was traced in the absence (trace a) or presence (trace b) of 100 U of NCX1 inhibitor. The Na_o-dependent Ca-efflux was detected by monitoring the fluorescence changes of extravesicular fluo-3 in the stopped-flow machine (see Materials and methods). Equal volumes (50 μl) of Ca-loaded vesicles inhibitor (syringe A) were mixed with NaCl/BAPTA/fluo-3 buffer (syringe B) to give a final concentration of 160 mM NaCl. The NCX1 inhibitor was included in syringe B. Each trace represents an average of seven independent mixings (see Materials and methods).

90–95%, meaning that both the forward and reverse modes of Na/Ca exchange are subject to nearly complete inhibition under given conditions. However, the addition of 500 U into the assay medium has no detectable effect either on the Na,K-ATPase (Fig. 3A) or the Ca-ATPase activity (Fig. 4A). By comparing the dose–response effects one can estimate that the endogenous NCX1 inhibitor has at least 10^3-fold higher potency for NCX1 inhibition as compared to the Na,K-ATPase or Ca-ATPase. In reality, the selectivity index for inhibition (NCX1 vs. Na,K-ATPase or Ca-ATPase) could be much higher. More accurate quantification of the selectivity index is somewhat difficult to perform today because the available amounts of purified NCX1 inhibitor are quite limited at this moment.

In general, the inhibition of Na,K-ATPase affects the function of NCX1 in indirect way. This is because the elevated levels of intracellular sodium can reduce the driving force for Ca-extrusion via NCX1, which in turn may result in enhancement of muscle contractility. Although the inhibition of Na,K-ATPase can potentially induce the positive inotropic effect, the high levels of sodium may upset basal ion-homeostasis in myocyte, thereby causing a general tissue toxicity (so called digitalis induced poisoning). This seems to be not the case for the endogenous inhibitor of NCX1 [18], presumably because the inhibition of Na/Ca exchange perhaps does not elevate the intracellular Na-levels (in contrast to the Na,K-ATPase, the Na/Ca exchanger is not responsible for controlling the Na-homeostasis in the cell). Therefore, a direct inhibition of NCX1 might have a certain advantage versus digitalis like compounds in terms of potential toxicity. This issue requires more dedicated investigation, because the relevant conclusions could be important for development of new drugs.

In most leaving cells (including the myocytes), the SL Ca-ATPase is responsible for creating and maintenance of the Ca-gradient across the cell membrane [1,2]. A major difference in the physiological role of NCX1 and SL Ca-ATPase is that the Ca-pump keeps the resting levels of cytosolic calcium, while the Na/Ca exchanger regulates the transient levels of calcium during the action potential [1–5]. The fact that the endogenous inhibitor of NCX1 does not affect the SL Ca-ATPase supports the idea that this regulatory factor might be involved in controlling the transient (systolic) rather the resting (diastolic) levels of cytosolic calcium. This proposal requires a careful examination of NCX1 inhibitor for its effects on transient and resting levels of cytosolic calcium in intact myocytes.

In light of present consideration, we conclude that the endogenous inhibitor of NCX1 does not resemble the properties of digitalis like compound and certainly, it cannot target the Na,K-ATPase or Ca-ATPase with high potency. Besides the strong inotropic effect of the endogenous inhibitor of NCX1, it can also suppress the ectopic contractions of ventricle muscle strips (unpublished data). This suggests that the NCX1 inhibitor may also have an antiarrhythmic potency. Thus, it is possible that newly found endogenous inhibitor of NCX1 may have capacity to regulate both the arrhythmogenic and contractile disorders. Although the potential outcome of this seems to be very encouraging for novel pharmacological intervention, one has to be very careful not to jump to conclusions because the related mechanisms have not been resolved yet under physiologically related conditions. For example, the effects of NCX1 inhibitor on forward and reverse modes of NCX1 currents must be tested in intact myocytes. This kind of tests could be even more interesting for comparison of the normal and

diseased myocytes (hypertrophy, heart failure, etc.) because the forward/reverse current balance is dramatically altered in cardiac disease [3–5].

Although the present studies provide no evidence for interaction of the endogenous NCX1 inhibitor with primary ion-pumps, this is only the beginning for systematic characterization of selectivity properties. It is obvious that multidisciplinary approaches must be applied for examining a possible interaction of the endogenous inhibitor of NCX1 with other ion-transport systems involved in Ca-fluxes and action potential. This includes, of course, the testing of effects on action potential duration and Ca-transients appearance as well as on major ion-currents involved in action potential. An application of the transgenic NCX1 mice could be another complementary approach for selectivity studies [27]. The advantage of this system is that "nonspecific" effects (induced by any NCX1 inhibitor) can be identified by recording the altered transients of cytosolic calcium in heart tubes obtained from the NCX1 knockout mice.

Acknowledgments

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