A Novel Isothiourea Derivative Selectively Inhibits the Reverse Mode of Na\(^+\)/Ca\(^{2+}\) Exchange in Cells Expressing NCX1*

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No.7943 ([2]-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate), a selective inhibitor of the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1), has been newly synthesized. It dose-dependently inhibited Na\(^+\)-dependent 45Ca\(^{2+}\) uptake and Na\(^+\)-dependent [Ca\(^{2+}\)]\(_i\) increase in cardiomyocytes, smooth muscle cells, and NCX1 transfected fibroblasts (IC\(_{50}\) = 1.2–2.4 μM). Inhibition was observed without prior incubation with the agent and was completely reversed by washing cells with buffer for 1 min. Interestingly, No.7943 was much less potent in inhibiting Na\(^+\)-dependent 45Ca\(^{2+}\) efflux and Na\(^+\)-induced [Ca\(^{2+}\)]\(_i\), decline (IC\(_{50}\) = >30 μM), indicating that it selectively blocks the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange in intact cells. In cardiac sarcosomale preparations consisting mostly of inside-out vesicles, the agent inhibited Na\(^+\)-dependent 45Ca\(^{2+}\) uptake and Na\(^+\)-dependent 45Ca\(^{2+}\) efflux with similar, but slightly lower, potencies (IC\(_{50}\) = 5.4–13 μM). Inhibition was noncompetitive with respect to Ca\(^{2+}\) and Na\(^+\) in both cells and sarcosomale vesicles. These results suggest that No.7943 primarily acts on external exchanger site(s) other than the transport sites in intact cells, although it is able to inhibit the exchanger from both sides of the plasma membrane. No.7943 at up to 10 μM does not affect many other ion transporters nor several cardiac action potential parameters. This agent at these concentrations also did not influence either diastolic [Ca\(^{2+}\)]\(_i\), or spontaneous beating in cardiomyocytes. Furthermore, No.7943 markedly inhibited Ca\(^{2+}\) overload into cardiomyocytes under the Ca\(^{2+}\) paradox conditions. Thus, No.7943 is not only useful as a tool with which to study the transport mechanism and physiological role of the Na\(^+\)/Ca\(^{2+}\) exchanger but also has therapeutic potential as a selective blocker of excessive Ca\(^{2+}\) influx mediated via the Na\(^+\)/Ca\(^{2+}\) exchanger under pathological conditions.

The Na\(^+\)/Ca\(^{2+}\) exchanger is a major regulator of [Ca\(^{2+}\)]\(_i\) in excitable as well as in many nonexcitable cells (1, 2). The exchanger catalyzes bidirectional electrogenic exchange of Na\(^+\) for Ca\(^{2+}\) across the plasma membrane, its direction being determined by the magnitude and orientation of electrical and chemical ion gradients. The exchanger works in concert with other cellular Ca\(^{2+}\) transporters including the sarcoendoplasmal Ca\(^{2+}\) pump and Ca\(^{2+}\) channels and intracellular Ca\(^{2+}\) quiescence and release systems. Thus the function of the exchanger under physiological or pathological conditions is often difficult to define, because the membrane potential or intracellular concentrations of Na\(^+\) and Ca\(^{2+}\) may vary in different cell types and change in response to agonist or electrical stimulation.

Recent molecular cloning studies have revealed that the Na\(^+\)/Ca\(^{2+}\) exchanger isoforms expressed in various cell types are highly homologous to the cardiac clone and are the product of the same gene (NCX1) (3–5). These isoforms, however, differ in a small region near the carboxyl end of the large central loop, which is due to alternative splicing (4, 5). In brain and skeletal muscle, a Na\(^+\)/Ca\(^{2+}\) exchanger isoform that is a product of a different gene (NCX2) is also expressed (6).

The physiological role of the Na\(^+\)/Ca\(^{2+}\) exchanger has been studied most extensively in cardiac muscle. During each action potential, the exchanger rapidly extrudes the Ca\(^{2+}\) that has entered the cardiomyocytes via the sarcoendoplasmal L-type Ca\(^{2+}\) channels to trigger the release of Ca\(^{2+}\) from the SR (7, 8). In addition, the exchanger has been shown to play a much greater role than the sarcoendoplasmal Ca\(^{2+}\) pump in the slow extrusion of Ca\(^{2+}\) from cardiomyocytes during diastole or under resting conditions (7, 9). On the other hand, the exchanger appears capable of bringing Ca\(^{2+}\) into cardiomyocytes during cardiac depolarization, although triggering the release of Ca\(^{2+}\) from the SR via the exchanger is much less efficient than via the L-type Ca\(^{2+}\) channels (10). Under pathological conditions such as ischemia-associated reperfusion injury, the exchanger is thought to cause Ca\(^{2+}\) overload of cardiomyocytes due to an increase in [Na\(^+\)]\(_i\) (11, 12). In other cell types, including nonexcitable cells such as kidney cells, however, the specific contribution of the exchanger to the [Ca\(^{2+}\)]\(_i\) regulation has been difficult to determine, because of the relatively low density of the exchanger in the plasma membranes of these cells and the lack of a specific inhibitor.

A potent and selective inhibitor of the Na\(^+\)/Ca\(^{2+}\) exchanger, if available, should be extremely useful to study the reaction mechanism of Na\(^+\)/Ca\(^{2+}\) exchange and to clarify its physiological and pathophysiological roles. Moreover, such an inhibitor may serve as a therapeutic agent by virtue of its inotropic, cardioprotective, antiarrhythmic, or antihypertensive effects. Although a variety of natural products, synthetic organic compounds, and inorganic cations have been tested for their ability to inhibit the exchanger (13), few selective inhibitors exist.

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†The abbreviations used are: [Ca\(^{2+}\)]\(_i\), intracellular Ca\(^{2+}\) concentration; [Ca\(^{2+}\)]\(_e\), extracellular Ca\(^{2+}\) concentration; Na\(^+\), intracellular Na\(^+\); Na\(^+\)-, extracellular Na\(^+\); SR, sarcoplasmatic reticulum; XIP, exchanger inhibitory peptide; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; BSA, bovine serum albumin; BSS, balanced salt solution; DHP, 1,4-dihydropyridine; Mops, 4-morpholinepropanesulfonic acid.

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Amiloride analogues such as 3',4'-dichlorobenzamil inhibit Na\(^+\)/Ca\(^{2+}\) exchange at micromolar concentrations, but they exert a cytotoxic effect by inhibiting a number of other ion transporters and cell metabolism (14–16). On the other hand, XIP, a synthetic peptide derived from the amino acid sequence highly specific (17) and has thus been used in previous studies (18, 19). However, XIP is highly cationic and interacts with calmodulin. With the latter property, it modulates activities of calmodulin-activated enzymes such as the sarcolemmal Ca\(^{2+}\)-ATPase (17). Since XIP does not seem to permeate through the cell membrane but acts from the cytoplasmic surface, its use is significantly limited. Therefore, development of a new potent inhibitor that is selective for the Na\(^+\)/Ca\(^{2+}\) exchanger in vitro is highly desired.

We report here that a newly synthesized compound, 2-[2-[4-(3-nitrobenzoyl)phenethyl]amino]thioureacarbamoyl methanesulfonate (designated No.7943), is a potent and selective inhibitor of the Na\(^+\)/Ca\(^{2+}\) exchanger. This compound (Fig. 1) has been identified by screening a compound library for inhibition of Na\(^+\)/Ca\(^{2+}\) exchange into isolated cardiac sarcolemmal vesicles. Surprisingly, this compound selectively inhibits the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange in intact cells. We also describe that this compound prevents the excess Ca\(^{2+}\) influx evoked by the Ca\(^{2+}\) paradox, which has been widely studied as an experimental model for Ca\(^{2+}\) overloading in cardiomyocytes.

**Experimental Procedures**

**Cell Cultures**—Primary cultures of neonatal rat cardiomyocytes were prepared by the method described previously (20). Briefly, hearts from 1- or 2-day-old Wistar rats were minced, and cells were dissociated with 0.1% trypsin in buffer A (20 mM Hepes/Tris (pH 7.4), 137 mM NaCl, 5.36 mM KCl, 0.81 mM MgSO\(_4\), 0.44 mM KH\(_2\)PO\(_4\), 0.34 mM Na\(_2\)HPO\(_4\), and 5.55 mM glucose) containing no added Ca\(^{2+}\). After centrifugation, the pellet was resuspended in DMEM (Life Technologies, Inc.) supplemented with 5% heat-inactivated FCS, 1.5 mM vitamin B\(_6\), 1 mg/ml insulin, 5 \(\mu\)g/ml transferrin, 50 units/ml penicillin, and 50 \(\mu\)g streptomycin. Dispersed cells were placed in 150-mm dishes (Falcon) for 1 h, and non-attached cells were seeded onto polystyrene dishes or onto Mycal. Dispersed cells were placed in 150-mm dishes (Falcon) for 1 h, insulin, 5 \(\mu\)M, 5 \(\times\) 10\(^5\) cells (Figs. 1 and 2), 10 mM Hepes/Tris (pH 7.4), 146 mM NaCl, 4 mM KCl, 2 mM MgCl\(_2\), 1 mM CaCl\(_2\), 10 mM glucose, and 0.1% BSA) containing 1 mM ouabain and 10 \(\mu\)M monensin. Na\(^+\)-dependent Ca\(^{2+}\) uptake was initiated by switching the medium to Na\(^+\)-free BSS containing equimolar chloride for normal BSS (10 mM Hepes/Tris (pH 7.4), 137 mM NaCl, 1 mM CaCl\(_2\), 10 mM glucose, and 0.1% BSA) containing 1 mM ouabain and 10 \(\mu\)M monensin. After a 15- or 30-s incubation, Na\(^+\)-dependent Ca\(^{2+}\) uptake was stopped by washing cells four times with an ice-cold solution containing 10 mM Hepes/Tris (pH 7.4), 120 mM choline chloride, and 10 mM LaCl\(_3\). Cells were solubilized with 0.1 N NaOH, and aliquots were taken for determination of radioactivity and protein. Na\(^+\)-dependent Ca\(^{2+}\) uptake was estimated by subtracting Na\(^+\)-free BSS from that in Ca\(^+\)-free BSS. Na\(^+\)-dependent Ca\(^{2+}\) uptake from cells in a 35-mm dish was assayed as described previously (24). Cells were equilibrated with Ca\(^{2+}\) by incubating them at 37 °C for 4 h in 1 ml of BSS containing 740 kBq of \(^{45}\)Ca\(^{2+}\). After rinsing cells six times with Ca\(^{2+}\)- and Na\(^+\)-free BSS for 1 min, \(^{45}\)Ca\(^{2+}\) efflux was measured in Ca\(^{2+}\) and Na\(^+\)-free BSS or in Ca\(^{2+}\)-free BSS. Measurement of \([Ca^{2+}]_{\text{i}}\) was monitored using fura-2 as a fluorescent Ca\(^{2+}\) indicator. Cells cultured on glass coverslips were loaded with 4 \(\mu\)M fura-2-acetoxymethyl ester for 20 min at 37 °C in buffered solutions containing 1 mM Ca\(^{2+}\) and 0.1% BSA either in normal BSS or in BSS (for smooth muscle cells and transfected CCL39 cells). Preloaded cells were then washed twice with the same medium. Glass coverslips were fixed to a mount that was diagonally inserted into a cuvette filled with 2.2 ml of the particular medium. The fluorescence signal was monitored and \([Ca^{2+}]_{\text{i}}\), calculated as described previously (25).

**Measurement of Na\(^+\)/Ca\(^{2+}\) Exchange in the Sarcolemmal Vesicles**—Sarcolemmal vesicles were prepared from dog ventricular muscle according to Jones (26). Na\(^+\)-dependent Ca\(^{2+}\) uptake into vesicles was measured essentially as described previously (27). Briefly, 5 \(\mu\)l of Na\(^+\)-loaded vesicles (1–2 mg/ml) was rapidly diluted into 0.25 ml of uptake medium (20 mM Mops/Tris (pH 7.4), 160 mM KCl, 5–80 \(\mu\)M \(^{45}\)CaCl\(_2\) (10 kBq), and 0.5 \(\mu\)M ouabain) at 37 °C. The reaction was stopped at 1.5 s by adding 4 ml of ice-cold washing medium (160 mM KCl and 1 mM LaCl\(_3\)). Vesicles were collected on a glass fiber filter and washed twice with the same medium. Blank vesicles were obtained by measuring \(^{45}\)Ca\(^{2+}\) uptake in medium containing NaCl instead of KCl. These blanks were subtracted from all data points to correct for Na\(^+\)-independent Ca\(^{2+}\) uptake. Na\(^+\)-dependent Ca\(^{2+}\) efflux was quantitated by measuring Na\(^+\)-induced \(^{45}\)Ca\(^{2+}\) loss from vesicles that had been preloaded with Ca\(^{2+}\) by Na\(^+\)-dependent Ca\(^{2+}\) uptake (28). Briefly, Na\(^+\)-loaded vesicles (5 \(\mu\)l) were diluted with 0.5 ml of the uptake medium containing 10 \(\mu\)M \(^{45}\)CaCl\(_2\) for 2 min at 37 °C. Ca\(^{2+}\)-efflux was then initiated by addition of 0.5 ml of efflux medium (20 mM Mops/Tris (pH 7.4), 120–160 mM KCl, 0.2 mM EGTA, and 0–40 mM NaCl). The reaction was stopped 10 s later by addition of 5 ml of NaCl. Blank vesicles were obtained by measuring \(^{45}\)Ca\(^{2+}\) loss in the efflux medium containing no NaCl.

**Assays of Other Ion Transporters**—L-type Ca\(^{2+}\) channel activity was assayed by measuring DHP-sensitive \(^{45}\)Ca\(^{2+}\) uptake into cultured smooth muscle cells as described previously (25). Briefly, cultured smooth muscle cells in 24-well dishes were preincubated at 37 °C for 30 min in 0.5 ml of normal BSS. To initiate \(^{45}\)Ca\(^{2+}\) uptake, cells were rinsed with BSS containing 1 mM (370 kBq) \(^{45}\)CaCl\(_2\) in the presence or absence of 1 mM (+)-PN203–110. After 2 min, \(^{45}\)Ca\(^{2+}\) uptake was stopped, and radioactivity and protein were determined. DHP-sensitive \(^{45}\)Ca\(^{2+}\) uptake was estimated by subtracting \(^{45}\)Ca\(^{2+}\) uptake in the presence of (+)-PN203–110 from that in the absence of (+)-PN203–110. L-Type Ca\(^{2+}\) exchange activity was assayed by measuring 5-(N-ethyl-N-isopropyl)amiloride-sensitive \(^{45}\)Na\(^+\) uptake as described previously (29). Cultured cardiomyocytes in 24-well dishes were preincubated with BSS containing 30 mM NH\(_4\)Cl for 30 min at 37 °C and subsequently washed twice with Na\(^+\)-free BSS for 40 s. \(^{45}\)Na\(^+\) uptake was then initiated by adding the Na\(^+\)-free BSS containing 1 mM (37 kBq) \(^{22}\)NaCl, 1 mM ouabain, and either 0 or 5 \(\mu\)l 5-(N-ethyl-N-isopropyl)amiloride. After 40 s, cells were washed four times with an ice-cold phosphate-buffered saline to stop \(^{45}\)Na\(^+\) uptake. Passive \(^{22}\)Na\(^+\) uptake into cultured cardiomyocytes was measured for 30 min at 37 °C in BSS containing 22NaCl (740 kBq/ml) and 1 mM ouabain.

**Na\(^+\),K\(^{-}\)**-ATPase activity was measured by incubating cardiac sarcolemmal vesicles (100 \(\mu\)g) for 20 min at 37 °C in a 1-mL reaction medium containing 20 mM Hepes/Tris (pH 7.4), 160 mM NaCl, 1 mM KCl, 5 mM MgCl\(_2\), 3 mM Na\(_2\)ATP, and 1 mM EGTA. The reaction was stopped by addition of 10% trichloroacetic acid, and Pi liberated was determined (30). The difference between activities in the presence and absence of 0.2 mM ouabain was taken as Na\(^+\),K\(^{-}\)-ATPase activity.

Ca\(^+\)-ATPase activity was measured using cardiac sarcolemmal vesicles or SR vesicles that were prepared from dog ventricular muscle.
The ATPase reaction was performed at 37°C with 50 μg of sarcolemmal vesicles for 20 min or with 10 μg of SR vesicles for 1 min in 0.5 ml of standard medium (20 mM Hepes/Tris [pH 7.2], 100 mM KCl, 5 mM MgCl₂, 0.1 mM CaCl₂, or 1 mM EGTA, 1 mM ATP, 2 mM phosphoenolpyruvate, 0.2 mg/ml pyruvate kinase, and 2 μM A23187), to which 5 μg/ml calmodulin, 1 μM thapsigargin, and 1 mM ouabain were added further when ATP hydrolysis by sarcolemmal vesicles was measured. After the reaction was terminated by adding 3 N HCl (0.1 ml) containing 2.5 mM 2,4-dinitrophenyl hydrazine, pyruvate produced was determined as described previously (32). The difference between activities in the presence and absence of CaCl₂ was taken as Ca²⁺-ATPase activity.

**Measurement of Action Potential**—Cardiac action potential was measured according to the standard method (33). Briefly, papillary muscle bundles were mounted into a chambered organ bath and superfused with Tyrode’s solution (137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.5 mM NaH₂PO₄, 11.9 mM NaHCO₃, and 5.5 mM glucose, gassed by 95% O₂/5% CO₂) at 10 ml/min at 36°C. Muscle preparations were stimulated at a constant rate of 2 Hz through bipolar electrodes with square-wave pulses of 0.5 ms and an intensity 2 times above threshold. Transmembrane potentials were measured by a high input impedance preamplifier (MEZ-7200, Nihon Kohden), displayed on dual beam oscilloscope (VC-11, Nihon Kohden), and stored on videotape recorder. The maximum rate of rise of the action potential (Vmax) was obtained by an electronic differentiator with linear differentiation.

**Protein Determination**—Protein was measured by the modified Lowry method (34) with BSA as a standard.

**Statistical Analysis**—Data are expressed as the means ± S.E. Differences for multiple comparisons were analyzed by one-way analysis of variance followed by the Dunnett’s test. Values of p < 0.05 were considered statistically significant.

**Materials**—No.7943 (2’-3’-4’-(4-nitrobenzoyloxy)phenethylisothiouracil methanesulfonate), 3’-4’-dichlorobenzamil, and 5-N-ethyl-N-isopropylamiloride were synthesized by the New Drug Research Laboratories, Kanebo Ltd. XIP (RLLFYKYYKYYKRYAGKQR) was synthesized by the Peptide Institute. ⁴⁵CaCl₂ and ²²NaCl were purchased from DuPont NEN. Ouabain, monensin, valinomycin, A23187, verapamil, nicardipine, and BSA (fatty acid-free) were from Sigma. Thapsigargin, cytochrome arabinoside, and calmodulin were from Wako Pure Chemical Co. Fura-2/acetoxymethyl ester was obtained from Dojindo Laboratories. (+)-PN200-110 was from Sandoz Ltd. All other chemicals were of analytical grade.

**RESULTS**

**Effect of No.7943 on Na⁺-dependent Ca²⁺ Influx**—No.7943 dose-dependently inhibited Na⁺-dependent ⁴⁵Ca²⁺ uptake into rat cardiomyocytes, rat aortic smooth muscle cells, and cardiac NCX1-transfected CCL39 cells (Fig. 2A). The IC₉₀ values for individual cell types were 2.4 ± 0.3, 2.0 ± 0.1, and 1.6 ± 0.2 μM (n = 3), respectively, the complete inhibition occurring at ≈30 μM of this agent. Thus, the inhibitory potency of No.7943 was very similar among these cell types. Under identical conditions, 10 μM 3’-4’-dichlorobenzamil inhibited Na⁺-dependent ⁴⁵Ca²⁺ uptake into rat cardiomyocytes by 30 ± 5% (n = 3). At 30 μM, however, 3’-4’-dichlorobenzamil exhibited cytotoxicity, causing cell rounding and detachment from dishes, whereas the same concentration of No.7943 did not exert such cytotoxicity. In contrast, XIP did not affect the Na⁺-dependent ⁴⁵Ca²⁺ uptake into cardiomyocytes at concentrations up to 100 μM. Of note, the inhibitory potency of No.7943 was identical whether ⁴⁵Ca²⁺ uptake was measured for 15 s in cardiac NCX1-transfected CCL39 cells before preincubation with this agent for 5 min (Fig. 2A) or without such preincubation (IC₉₀ = 2.1 ± 0.4 μM (n = 3)). No.7943 at up to 30 μM did not affect ⁴⁵Ca²⁺ uptake into nontransfected CCL39 cells (data not shown), which is consistent with the lack of detectable Na⁺-dependent ⁴⁵Ca²⁺ uptake in these cells (23).

From the data summarized in Table I, we examined the effect of No.7943 on the rate of Na⁺-dependent ⁴⁵Ca²⁺ uptake into NCX1-transfected cells measured as a function of Ca²⁺ concentration (Fig. 2B). The observed Ca²⁺ concentration dependences obeyed Michaelis-Menten kinetics. No.7943 at 1 and 3 μM decreased Vmax to 7.2 ± 0.2 and 4.5 ± 0.3 nmol/mg/15 s (n = 3), respectively, compared with the control value of 8.8 ± 0.1 nmol/mg/15 s (n = 3). No.7943 did not affect the K₉₀ (0.14 ± 0.01 mm (n = 3) for control and 0.15 ± 0.01 mm (n = 3) for cells treated with 1 or 3 μM No.7943). We also examined the effect of No.7943 on Na⁺-dependent inhibition of Na⁺-dependent ⁴⁵Ca²⁺ uptake into NCX1-transfected cells (Fig. 2C). Na⁺ inhibited Na⁺-dependent ⁴⁵Ca²⁺ uptake in a concentration-dependent manner. The IC₅₀ for Na⁺ was similar in control cells (69 ± 3.1 mm, n = 3) and in cells treated with 1 μM (68 ± 3.4 mm, n = 3) or 3 μM (67 ± 2.2 mm, n = 3) No.7943. Thus, the extent of inhibition by No.7943 was similar at different Na⁺ concentrations. These results indicate that No.7943 does not compete with Ca²⁺ for Na⁺.

Fig. 3 shows the inhibitory effect of No.7943 on Na⁺-dependent increase in [Ca²⁺], measured in aortic smooth muscle cells in the presence of 10 μM verapamil. A transient increase of [Ca²⁺] was induced in these cells by switching the medium from a Na⁺-containing to a Na⁺-free solution for 2 min. The [Ca²⁺]ₚ as estimated from the plot of the initial rate of rise in [Ca²⁺] versus the No.7943 concentration, was 1.6 ± 0.2 μM (n = 3). This is very close to the IC₅₀ values obtained from the ⁴⁵Ca²⁺ uptake measurements (see above). Interestingly, the
inhibition by this agent completely disappeared after washing cells with fresh medium for 1 min (Fig. 3), indicating that the effect is fully reversible. The transient nature of [Ca\textsuperscript{2+}]\textsubscript{i}, rise observed here might have arisen, at least in part, from a time-dependent decrease in Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange activity, which was caused by the exchange-induced reduction in [Na\textsuperscript{+}], and development of deeper negative membrane potential, as well as by rapid removal of cytoplasmic Ca\textsuperscript{2+} by the sarcolemmal and SR Ca\textsuperscript{2+} pumps. In cardiomyocytes, a similar IC\textsubscript{50} value (1.2 ± 0.1 \mu M, n = 3) was also obtained for the inhibition by No.7943 of the initial rate of Na\textsuperscript{+} \textsuperscript{-} dependent increase in [Ca\textsuperscript{2+}]\textsubscript{i}. In this experiment, cardiomyocytes were preincubated at 37 °C for 20 min with buffer A containing 137 mM NaCl, 1 mM ouabain, 10 \mu M verapamil, and 0.1 mM Ca\textsuperscript{2+}, and then an increase in [Ca\textsuperscript{2+}]\textsubscript{i} was evoked by the addition of 1 mM Ca\textsuperscript{2+} to the medium. A steady increase in [Ca\textsuperscript{2+}]\textsubscript{i} was observed at least for 2 min under these conditions (data not shown).

**Effect of No.7943 on Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} Extrusion**—We examined the effect of No.7943 on 45Ca\textsuperscript{2+} efflux from 45Ca\textsuperscript{2+}-labeled NCX1-transfected cells in a Ca\textsuperscript{2+}- and Na\textsuperscript{+}-free medium or in a Ca\textsuperscript{2+}-free medium containing 146 mM Na\textsuperscript{+} (Fig. 4). The cells were treated with thapsigargin for 20 s to raise [Ca\textsuperscript{2+}]\textsubscript{i}. In control cells, the rate of Na\textsuperscript{+} \textsuperscript{-} independent 45Ca\textsuperscript{2+} efflux was 0.22 ± 0.03 nmol/mg/20 s (n = 4), whereas the rate of Na\textsuperscript{+} \textsuperscript{-} dependent 45Ca\textsuperscript{2+} efflux was 0.70 ± 0.13 nmol/mg/20 s (n = 4), as estimated by subtracting 45Ca\textsuperscript{2+} efflux in the absence of Na\textsuperscript{+} from that in the presence of Na\textsuperscript{+} \textsuperscript{-} dependent No.7943 at 10 and 30 \mu M did not affect Na\textsuperscript{+} \textsuperscript{-} independent 45Ca\textsuperscript{2+} efflux but decreased the rate of Na\textsuperscript{+} \textsuperscript{-} dependent 45Ca\textsuperscript{2+} efflux by 7 and 38%, respectively. This weak inhibition could not be increased by preincubation of cells with No.7943 for up to 30 min (data not shown). Thus only high doses of No.7943 inhibited Na\textsuperscript{+} \textsuperscript{-} dependent 45Ca\textsuperscript{2+} efflux.

We studied the effect of No.7943 on the time course of Na\textsuperscript{+} \textsuperscript{-} induced [Ca\textsuperscript{2+}]\textsubscript{i} decline in smooth muscle cells under conditions where the sarcolemmal and SR Ca\textsuperscript{2+} pumps were inhibited by thapsigargin (1 \mu M) and a Ca\textsuperscript{2+} - and Na\textsuperscript{+} -free, high pH (pH 8.8) medium containing 20 mM MgCl\textsubscript{2} (24, 35). Under these conditions, [Ca\textsuperscript{2+}]\textsubscript{i} increased to a relatively high level (700–800 nm), and we observed spontaneous decline of [Ca\textsuperscript{2+}]\textsubscript{i}, (30 ± 3 nm/10 s, n = 4) (Fig. 5). Addition of 50 mM Na\textsuperscript{+} accelerated the [Ca\textsuperscript{2+}]\textsubscript{i} decline with a resultant initial rate of 157 ± 7 nm/10 s (n = 4), whereas addition of 50 mM choline chloride had no effect. No.7943 at 10 and 30 \mu M decreased the Na\textsuperscript{+} \textsuperscript{-} dependent portion of [Ca\textsuperscript{2+}]\textsubscript{i} decline by 12 ± 5 and 37 ± 2% (n = 4), respectively, although the same concentrations of this agent did not affect the background [Ca\textsuperscript{2+}]\textsubscript{i}, decline (Fig. 5).

**Inhibition of Na\textsuperscript{+}/Ca\textsuperscript{2+} Exchange in Sarcolemmal Vesicles by No.7943**—The inhibitory effect of No.7943 on Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange was studied using cardiac sarcolemmal vesicles. As in cells, No.7943 completely and dose-dependently inhibited the initial rate of Na\textsuperscript{+} \textsuperscript{-} dependent Ca\textsuperscript{2+} uptake versus extravascular [Ca\textsuperscript{2+}], (Fig. 6B) revealed that 5 \mu M No.7943 decreased the V\textsubscript{max} to 11 ± 0.2 nmol/mg/1.5 s (n = 3) from the control value of 22 ± 0.6 nmol/mg/1.5 s (n = 3), whereas it did not affect the K\textsubscript{m} for Ca\textsuperscript{2+} (35 ± 4.3 \mu M for control and 34 ± 2.8 \mu M for the presence of the agent). The results indicate that inhibition is noncompetitive with respect to Ca\textsuperscript{2+}. In contrast, inhibition by XIP was incomplete (about 70%) with an IC\textsubscript{50} value of 1.0 ± 0.1 \mu M (n = 3) (Fig. 6B).

![Graph](Image)
Inhibitor of Na\(^+\)/Ca\(^{2+}\) Exchange

**TABLE I**

| Effect of No. 7943 on ion transporters |
|--------------------------------------|
| **No. 7943**                          |
| Control                              |
| 10 \(\mu\)M                         |
| 30 \(\mu\)M                         |
| Na\(^+/\)H\(^+\) exchange (nmol/mg/min) | 6.1 ± 0.3  6.0 ± 0.2  5.9 ± 0.1 |
| DHP-sensitive 45Ca\(^{2+}\) uptake (nmol/mg/2 min) | 2.9 ± 0.3  2.5 ± 0.3  1.9 ± 0.1* |
| Passive Na\(^+\) uptake (nmol/mg/30 min) | 310 ± 8.2  313 ± 18  293 ± 4.9 |
| Sarcolemmal Ca\(^{2+}\)-ATPase (nmol/mg/20 min) | 191 ± 14  203 ± 4.6  171 ± 14 |
| SR Ca\(^{2+}\)-ATPase (nmol/mg/min) | 828 ± 8.2  871 ± 16  771 ± 28 |
| Na\(^+\),K\(^+\)ATPase (\(\mu\)mol/mg/h) | 68 ± 4.1  67 ± 2.0  64 ± 2.5 |

*\(p < 0.05\) compared with control.

FIG. 6. Inhibition of Na\(^+\)/Ca\(^{2+}\) exchange by No. 7943 in sarcolemmal vesicles. A, Na\(^+\)-dependent 45Ca\(^{2+}\) uptake into cardiac sarcolemmal vesicles was measured in the presence of 10 \(\mu\)M KCl for 1.5 s as a function of indicated concentrations of No. 7943 (○) or XIP (●). The data are presented as percentage of the control value. Each data point represents an average of triplicate determinations. B, Na\(^+\)-dependent 45Ca\(^{2+}\) uptake measured in uptake medium containing 5–80 \(\mu\)M 45Ca\(^{2+}\) in the absence (○) and presence of 5 \(\mu\)M No. 7943 (●) or 1 \(\mu\)M XIP (▲). The data are presented in an Eadie-Hofstee plot. C, effects of indicated concentrations of No. 7943 on Na\(^+\)-dependent 45Ca\(^{2+}\) efflux from cardiac sarcolemmal vesicles were measured for 10 s in the presence of 10, 20, and 40 mM NaCl (replacing KCl with equimolar NaCl). Na\(^+\)-dependent 45Ca\(^{2+}\) uptake into and Na\(^+\)-dependent 45Ca\(^{2+}\) efflux from sarcolemmal vesicles were performed as described under "Experimental Procedures."
Inhibitor of Na\(^{+}/Ca^{2+}\) Exchange

Fig. 7. Effects of No.7943 on diastolic [Ca\(^{2+}\)]\(_{i}\) (A) and on Ca\(^{2+}\) overload evoked by the Ca\(^{2+}\) paradox (B) in cardiomyocytes. Fura-2-loaded cardiomyocytes were equilibrated with buffer A containing 1 mM CaCl\(_2\) and 0.1% BSA for 20 min at 37 °C. A, No.7943 was then cumulatively added at 100-s intervals as indicated. B, cardiomyocytes were exposed to a Ca\(^{2+}\)-, Mg\(^{2+}\)-free buffer A containing 0.2 mM EGTA for 10 min and then to buffer A containing 1 mM Ca\(^{2+}\) and 0 (control), 3, or 10 \(\mu\)M No.7943. No.7943 was added 5 min before the repletion of Ca\(^{2+}\).

\(^{45}\)Ca\(^{2+}\) uptake and [Ca\(^{2+}\)]\(_{i}\) increase into rat cardiomyocytes, rat aortic smooth muscle cells, and cardiac NCX1-transfected cells (IC\(_{50}\) = 1.2–2.4 \(\mu\)M) (Figs. 2 and 3). In contrast, the same agent produced only a weak inhibitory effect on the Na\(^{+}/Ca^{2+}\) exchanger in NCX1-transfected but not in nontransfected CCL39 cells (23). No.7943 inhibited the Na\(^{+}/Ca^{2+}\) exchanger, because of the following. (i) In cardiomyocytes and smooth muscle cells, both Na\(^{+}\)-dependent increase and Na\(^{+}\)-dependent decrease in [Ca\(^{2+}\)]\(_{i}\) are due to the activity of the Na\(^{+}/Ca^{2+}\) exchanger (1). The IC\(_{50}\) values for No.7943 obtained here by [Ca\(^{2+}\)]\(_{i}\) measurements (1.2–1.6 \(\mu\)M) were almost identical to those from the \(^{45}\)Ca\(^{2+}\) flux measurements (2.0–2.4 \(\mu\)M) (Figs. 2A and 3, and see also “Results”). (ii) Na\(^{+}\) and Na\(^{+}\)-induced changes in both \(^{45}\)Ca\(^{2+}\) fluxes and [Ca\(^{2+}\)]\(_{i}\) were observed in NCX1-transfected but not in nontransfected CCL39 cells (23). No.7943 inhibited the Na\(^{+}\)-dependent \(^{45}\)Ca\(^{2+}\) uptake into NCX1-transfected CCL39 cells (Fig. 2A) but not \(^{45}\)Ca\(^{2+}\) uptake into nontransfected CCL39 cells (see “Results”). (iii) No.7943 potently inhibited both Na\(^{+}\)-dependent Ca\(^{2+}\) uptake into and Na\(^{+}\)-dependent Ca\(^{2+}\) efflux from sarcolemmal vesicles (IC\(_{50}\) = 5.4 and 11 to 13 \(\mu\)M, respectively) (Fig. 6, A and C). In intact cells, therefore, No.7943 exerts a much greater inhibitory effect on the Na\(^{+}/Ca^{2+}\) exchanger operating in the reverse mode as compared with the exchanger operating in the forward mode. Furthermore, there is no difference in the inhibitory potency of No.7943 in different exchanger isoforms from cardiac and smooth muscle cells.

No.7943 is an amphiphilic molecule with an isothiourea group whose pK\(_{a}\) is about 10. Thus this agent is protonated and cationic in most conditions (see Fig. 1). This positive charge on the isothiourea moiety seems to be essential for inhibitory activity, as its deletion renders this agent much less active.\(^2\)

No.7943 at up to 100 \(\mu\)M is soluble in aqueous buffers. Interestingly, inhibition by No.7943 was easily abolished by washing cells with fresh medium for 1 min (Fig. 3), indicating that the effect is completely reversible and that removal of the agent is relatively rapid. Furthermore, the inhibitory potency of No.7943 was almost identical with or without prior preincubation (see “Results”). We therefore conclude that the agent primarily acts from the extracellular side of the plasma membrane in intact cells under the conditions used in this study. It should be noted, however, that No.7943 potently inhibited Na\(^{+}\)-dependent Ca\(^{2+}\) uptake into cardiac sarcolemmal vesicles, when the latter was exposed to the agent for only 1.5 s (Fig. 6A). The agent thus appears able to inhibit exchange activity also from the cytoplasmic side of the membrane, because a majority of sarcolemmal vesicles used in this study seem to have had inside-out orientation as inferred from the extent of inhibition by XIP. XIP at 10 \(\mu\)M, which presumably is membrane-impermeable (17), inhibited Na\(^{+}\)-dependent Ca\(^{2+}\) uptake into sarcolemmal vesicles by 70% (Fig. 6A).

We examined the kinetics of inhibition by No.7943 of the reverse (Ca\(^{2+}\)- influx) mode of Na\(^{+}/Ca^{2+}\) exchange with respect to either Ca\(^{2+}\)\(_{o}\) or Na\(^{+}\)\(_{o}\) concentration using intact cells (Fig. 2, B and C). The exchanger inhibition was noncompetitive with Ca\(^{2+}\)\(_{o}\) and inhibition of Na\(^{+}\)-dependent \(^{45}\)Ca\(^{2+}\) uptake by Na\(^{+}\) was not influenced by 1 or 3 \(\mu\)M No.7943. Similarly, No.7943 inhibited Na\(^{+}\)-dependent \(^{45}\)Ca\(^{2+}\) uptake into sarcolemmal vesicles by reducing \(V_{\text{max}}\) without affecting the \(K_{m}\) for extravesicular Ca\(^{2+}\) (Fig. 6B). This vesicular exchange reactions (Na\(^{+}\)-dependent \(^{45}\)Ca\(^{2+}\) uptake and Na\(^{+}\)-dependent \(^{45}\)Ca\(^{2+}\) efflux) are mostly due to inside-out vesicles (17) and presumably equivalent to the forward and reverse modes of the exchange in intact cells. From all these results, we conclude that No.7943 does not affect the interaction of transport sites on the exchanger with Ca\(^{2+}\) or Na\(^{+}\) on either side of the plasma membrane and thus probably acts at site(s) distinct from these sites. It is striking that in intact cells the potency of No.7943 as a blocker of the reverse mode of Na\(^{+}/Ca^{2+}\) exchange is 15–25-fold greater compared with that for the forward mode. In contrast, a minimum difference was observed for the effect of No.7943 on the corresponding reactions in sarcolemmal vesicles (Fig. 6, A and C, and see above). Thus, the mode-specific inhibition by No.7943 was observed only in intact cells. Such a difference in the inhibitory pattern may be consistent with our view that the agent acts on the exchanger primarily from the extracellular side in intact cells, whereas it acts mainly from the cytoplasmic side in sarcolemmal vesicles. At present, however, we have no information about the mechanism by which this agent causes such a mode-specific inhibition of Na\(^{+}/Ca^{2+}\) exchange in intact cells.

No.7943 at 0.3–10 \(\mu\)M, while blocking the Na\(^{+}/Ca^{2+}\) exchanger-mediated Ca\(^{2+}\) influx into cells, did not significantly affect activities of other ion transporters such as Na\(^{+}/H^{+}\) exchanger, DHP-sensitive Ca\(^{2+}\) channels, sarcolemmal and SR Ca\(^{2+}\) ATPases, and Na\(^{+}\)-K\(^{+}\)ATPase, as well as passive Na\(^{+}\) permeability (Table I and Fig. 4). In addition, the same concentration range of No.7943 did not significantly alter the action potential parameters such as resting membrane potential, action potential amplitude, the maximum rate of rise of action potential (\(V_{\text{max}}\)), and action potential duration at 90% repolarization (see “Results”). However, No.7943 at a high concentration (30 \(\mu\)M) reduced the activities of voltage-dependent Na\(^{+}\) channels (measured as \(V_{\text{max}}\)) and DHP-sensitive Ca\(^{2+}\) channels, as well as the forward mode of the Na\(^{+}/Ca^{2+}\) exchange. In cultured

\(^{2}\) T. Iwamoto, S. Wakabayashi, and M. Shigekawa, unpublished observations.

\(^{3}\) T. Iwamoto and M. Shigekawa, unpublished results.
cardiomyocytes, No.7943 at up to 10 µM affected neither dia-
stolic [Ca<sup>2+</sup>], nor spontaneous beating (Fig. 7A), the latter being abolished by the Ca<sup>2+</sup> channel antagonist verapamil. In contrast, 30 µM No.7943 significantly increased the resting [Ca<sup>2+</sup>], (Fig. 7A). Thus, inhibition of Ca<sup>2+</sup> influx via the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger by low concentrations of No.7943 has virtually no effect on Ca<sup>2+</sup> mobilization and spontaneous beating in cultured cardiomyocytes. On the other hand, inhibition of Ca<sup>2+</sup> extrusion via the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger by a high concentration of this agent causes an increase in resting [Ca<sup>2+</sup>], probably due to the continued influx of Ca<sup>2+</sup> via verapamil-sensitive Ca<sup>2+</sup> channels. All these results indicate that No.7943 at relatively low concentrations is a selective inhibitor of the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger that only minimally affects cell ion metabolism. In this sense, No.7943 clearly is much superior to 3',4'-dichloro-
benzamil whose specificity is low (see the Introduction).

We explored the therapeutic potential of No.7943 by using the Ca<sup>2+</sup> paradox model (Fig. 7B). The Ca<sup>2+</sup> paradox has been studied as an experimental model for Ca<sup>2+</sup> overload in cardiomyocytes during the ischemia-associated reperfusion. The Na<sup>+</sup>/Ca<sup>2+</sup> exchanger operating in the Ca<sup>2+</sup> influx mode has been implicated in this mechanism. The same mode of the Na<sup>+</sup>/Ca<sup>2+</sup> exchange is also considered to be responsible for Ca<sup>2+</sup> overload during reperfusion after cardiac hypoxia (11, 12). We found that low concentrations of No.7943 were very effective in preventing Ca<sup>2+</sup> influx into cardiomyocytes and the resultant structural change under Ca<sup>2+</sup> paradox conditions (Fig. 7B and see “Results”). Importantly, this agent at the same low concentrations (up to 10 µM) also effectively blocks mechanical dysfunction of isolated perfused rat hearts that is caused by the ischemia/reperfusion or by hypoxia/reoxy-
egenol insult, whereas it had no effect on mechanical function of normal rat hearts<sup>4</sup> (36). Thus, No.7943 could have a therapeutic potential as a selective blocker of excessive Ca<sup>2+</sup> influx mediated via the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger under pathologi-
ical conditions, which include cardiac ischemia/reperfusion, hypoxia/reoxygenation, and possibly some forms of essential hypertension.

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