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# Advanced procedures for separation and analysis of low molecular weight inhibitor ($NCX_{IF}$) of the cardiac sodium–calcium exchanger ☆,☆☆

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**Abstract**

A low molecular weight inhibitor ($NCX_{IF}$) of the cardiac Na/Ca exchanger, isolated from the calf ventricle tissue, is capable of regulating the muscle strip's contractility and relaxation without involving the β-activation pathway. The structural analysis of $NCX_{IF}$ requires highly purified preparations that fulfill the demanding requirements for mass spectra and NMR analyses. No such preparation is yet available. To this end, new HPLC procedures were developed by a combination of the reverse phase, normal phase, and HILIC (hydrophilic liquid chromatography) techniques. The specific activity of $NCX_{IF}$ is $10^5$ times higher in the purified preparations (as compared to the crude extract) showing a 2–5% yield of total inhibitory activity and 20–100 μg content of final material. The purification yield reveals that 1 kg ventricle muscle contains 0.1–0.2 mg $NCX_{IF}$, meaning that the tissue concentrations of $NCX_{IF}$ may reach $10^{-7}$–$10^{-6}$ M. The diode-array scanning of purified preparations of $NCX_{IF}$ shows a homogeneous 3D peak with a maximal absorption at 202 nm. These spectral properties may represent a five-membered ring (e.g., proline, histidine) and/or simple chemical groups (like amine, carbonyl, ester, etc.), but not an aromatic ring or complex conjugates (alkyne, alkene, aldehyde, etc.). $NCX_{IF}$ does not respond to phenol/sulfur reagent, suggesting that it lacks reducing (aldo) sugar. $NCX_{IF}$ shows a faint response to fluorescamine, meaning that it may contain an amino group (or its derivative). It is believed that a combination of presently developed procedures with LC/MS and LC/MS/MS may provide a useful tool for structural analysis of $NCX_{IF}$.


*Keywords:* Calcium; Sodium–calcium exchange; Endogenous inhibitor; NCX1; Cardiac muscle

Two Ca-transport systems are responsible for removing the Ca from the cell, the cell membrane Ca-ATPase and the Na/Ca exchanger [1]. A partial contribution of each system to Ca-homeostasis depends on the functional characteristics of specific cell types [1,2]. In cardiomyocytes, the Na/Ca exchanger (NCX1) protein [3] extrudes nearly all the Ca that has entered into the cell during the action potential [4]. The Na/Ca exchange cycle involves a separate translocation of $Na^+$ and $Ca^{2+}$ ions [5], by exchanging $3Na^+$ ions for one $Ca^{2+}$ [6]. Although the Na/Ca exchanger can mediate the Ca-entry mode, the Ca-extrusion mode is the physiologically predominant one [7,8]. Altered conditions ($\uparrow[Na]_i$, $\downarrow[Ca]_i$-transients, prolonged action potential, and overexpressed NCX1) promote the Ca-entry mode via NCX1, which may represent a major concern for life-threatening arrhythmia [9,10]. In heart failure, down-regulation of SERCA2 and up-regulation of NCX1 may lead to "Ca-escape" resulting in contractile malfunction and arrhythmia [11–13].

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☆☆ *Abbreviations:* Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, Tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; FRCRCFa, cyclic hexapeptide Phe-Arg-Cys-Arg-Cys-Phe-$NH_2$ with intramolecular S—S bond; TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid; LC/MS, liquid chromatography coupled with MS; LC/MS/MS, liquid chromatography coupled with MS/MS; GC/MS/MS, gas chromatography coupled with mass spectroscopy.

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Moderate inhibition of overexpressed NCX1 may represent a major pharmacological breakthrough [14,15], but this intervention is hampered because of a poor understanding of the regulatory mechanisms and the ineffective performance of currently available NCX blockers. Synthetic blockers, like amiloride derivatives [16] and more recent methanesulfonate inhibitors, KB-R7943 [17] and SEA0400 [18], still need further improvement [19]. Despite the fact that the peptide blockers, XIP [20] and FRCRCFa [21,22], exhibit high potency and selectivity for NCX1 inhibition in patch–clamp experiments [23], their usage in intact physiological systems is restricted because they are cell-impermeable [24,25].

In searching for physiologically relevant regulators, we have detected and isolated an endogenous inhibitor ($NCX_{IF}$) of Na/Ca exchange, which is capable of modulating the muscle strip's contractility [26]. $NCX_{IF}$ can inhibit the forward or reverse modes of Na/Ca exchange as well as its partial reaction, the Ca/Ca exchange [26,29]. $NCX_{IF}$ can interact (within a few milliseconds) with the putative regulatory domain at the cytosolic side of sarcolemma vesicles [26,29]. The gel-filtration and mass spectra analyses reveal a small molecule (350–550 Da) [26–29] that has no relation to XIP [20] or phospholemman [30]. $NCX_{IF}$ neither inhibits the Na/K ATPase or SL Ca-ATPase activities [27] nor exhibits "digitalis-like" poisoning [26,28]. A modulation of muscle contractility by $NCX_{IF}$ does not involve the β-adrenergic pathway [28].

A molecular identification of $NCX_{IF}$ is still a major barrier for a breakthrough in the field. In fact, $NCX_{IF}$ is a low-abundant substance, which already makes it adverse for purification. However, a major problem is that $NCX_{IF}$ has extremely unfavorable physicochemical properties for separation and analysis. In general, small, polar, and uncharged compounds (such as $NCX_{IF}$) are poorly retained even on specially designed polar-embedded RP-HPLC columns [31]. Moreover, the ion suppression and detrimental matrix effects severely limit an exploration using advanced LC/MS and LC/MS/MS techniques [31–33]. In addition, aqueous phases are not conducive to achieving good spray conditions (especially for electron spray ionization), thereby resulting in low sensitive detection [32–34].

In our previous studies, the $NCX_{IF}$ activity was purified by using the gel filtration (Sephadex G-10), cation exchange (Dowex), reverse phase ($C_{30}$), and normal phase (TSK amide-80) columns [26]. Although the TSK amide-80 column shows high performance, the other columns were not good enough for fulfilling the analytical studies. To meet the demanding requirements for molecular analysis and functional studies, it was absolutely necessary to introduce more advanced procedures of the reverse-phase (RP), normal-phase (NP), and HILIC (hydrophilic interaction liquid chromatography) Chromatography. In the present study, we integrated the beneficial and complementary approaches of advanced HPLC techniques [31–34] to make possible an application of modern LC/MS and LC/MS/MS techniques. Therefore, the present studies may provide a firm basis for structural study of $NCX_{IF}$.

## Materials and methods

*Materials and reagents*. Protease inhibitors (PMSF, pepstatin, leupeptin, and aprotienin), Deoxyribonuclease I (type DN-25), fluorescamine, Dowex-50W1 × 8 (100–200 mesh), Dowex-AG1 × 4 (100–200 mesh), and laminin were obtained from Sigma (St. Louis, MO). Sephadex G-10 (fine) was from Pharmacia (Uppsala, Sweden). The glass microfiber filters (GF/C Whatman) were purchased from Tamar (Jerusalem, Israel). The Chelex 100 (ion-exchange resin) was obtained from Bio-Rad (USA). $^{45}CaCl_2$ (10–30 mCi/mg) was purchased from DuPont NEN (Boston, MA) or Perkin-Elmer (Monza, Italy). The scintillation cocktail Opti-Fluor was from Packard (Groningen, The Netherlands). All other reagents were of analytical or HPLC grade. Deionized water (18 MΩ/cm, Millipore System) was used for all solutions.

*Preparation of sarcolemma vesicles and assay of Na/Ca exchange.* The sarcolemma vesicles were obtained according to established procedures [21,35–37]. The vesicles (5–14 mg of protein/ml) were stored in 20 mM Mops/Tris, pH 7.4, and 0.25 M sucrose at −70 °C. Initial rates of $Na_i$-dependent $^{45}Ca$ uptake were in the range of 0.5–2 nmol Ca $mg^{-1}$ $s^{-1}$. No change in $Na_i$-dependent $^{45}Ca$ uptake activity was detected (at least for six months) for sarcolemma vesicles when stored at −70 °C. The Na/Ca exchange was measured by rapid dilution of Na- or Ca-loaded vesicles in the assay medium containing $^{45}Ca$ [21,36,37]. The assay medium (0.2–0.5 ml) contained 50 mM Mops/Tris, pH 7.4, 0.25 M sucrose, 40 μM $^{45}CaCl_2$ ($0.5–2.0 \times 10^6$ cpm/nmol) plus various amounts of purified fractions. Lipid and protein-free aliquots of tested fractions were lyophilized, dissolved in water, and corrected for pH (if necessary) by concentrated Tris/Mops buffer. At all stages of purification the concentrated fractions can be stored at −70 °C without a considerable loss of inhibitory activity of $NCX_{IF}$ (at least for 4–5 months). The samples (10–50 μl) of concentrated fractions were added to the assay medium about 20–30 min before the experiment. The $^{45}Ca$-uptake assay was initiated by diluting (20–50-fold) the $Na_i$- or $Ca_i$-loaded vesicles in the assay medium at 37 °C. The $^{45}Ca$ uptake ($t = 2–20$ s) was controlled electrically by rapid injection of quenching solution [6,21,35]. Quenched samples were filtered on the GF/C filters and subsequently counted by using a scintillation solution [21,22].

*Extraction and batch purification of $NCX_{IF}$.* $NCX_{IF}$ was extracted from calf ventricle muscle (26–29). Briefly, 6–12 kg of fresh calf ventricles (obtained from the nearest slaughterhouse) was freed from fat, sliced into small pieces, and lyophilized. The lyophilized powder was extracted with chloroform (1:1, v/v) at room temperature for 30–40 min. After filtration on gauze, the filtrate was discarded. The remaining slurry was extracted 5–6 times with 5–7 L portions of 95% ethanol. Next, the extract was filtered and evaporated in the hood to remove ethanol. The remaining aqueous phase was lyophilized to 300–400 ml and centrifuged at 20,000*g* for 30 min. The supernatant was lyophilized to ~100 ml and centrifuged at 50,000*g* for 30 min. Cold methanol (−20 °C) was added to the supernatant (1:5) and white precipitate was removed by filtration on Whatman 3MM. The filtrate was reduced to 100 ml by evaporation and ethanol was added to get a precipitate. This procedure was repeated until no precipitate appeared. After ethanol evaporation, the volume was adjusted to 150 ml by adding water and mixed by gentle stirring with 150 ml slurry of Dowex 2 × 8 (acetate form) for 30–40 min. The slurry was filtered on Whatman 3MM and the filter was flushed with water (5 × 50 ml). The effluent was mixed with 150 ml Dowex-50 W ($H^+$ form) by stirring for 30–40 min. After filtration on Whatman 3MM, the slurry was washed with water (5 × 50 ml). Afterwards, the collected effluent (reduced to 50–60 ml) was mixed with cold acetone (1:4) and remained for 24 h at −70 °C. A white precipitate was removed by filtration and the acetone was evaporated. The remaining aqueous fraction was stored at −70 °C until further use. Partially purified material (20–30 ml) was loaded onto the Sephadex G-10 column (5.5 × 70 cm) and eluted with water (2 ml/min). The collected fractions were analyzed for inhibition of Na/Ca exchange [26–29] and for peptide content [38] as well as reducing sugar [39]. Active fractions were collected, lyophilized, dissolved in deionized water, and stored at −70 °C until use.

*Chromatography.* The Waters HPLC System (Waters, Milford, USA), equipped with two M616A pumps, gradient maker, and sample injector