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An Unknown Endogenous Inhibitor of Na/Ca Exchange Can Enhance the Cardiac Muscle Contractility

Reuben Hiller, Chagit Shpak,[1] Gabriel Shavit, Beny Shpak, and Daniel Khananshvili[2]
Department of Physiology and Pharmacology, Sackler School of Medicine, Tel-Aviv University, Ramat-Aviv 69978, Israel

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The cardiac sarcolemma Na/Ca exchanger is a key system for controlling the intracellular calcium levels during the excitation–contraction coupling. Here, we test the hypothesis that the heart tissue contains a putative endogenous factor having a capacity to modulate the Na/Ca exchanger and muscle contractility. The concentrated cardiac extracts inhibit the Na_i- or Ca_i-dependent ^{45}Ca uptakes in isolated cardiac sarcolemma vesicles as well as the Na_o-dependent Ca efflux, monitored by extravesicular Ca probe fluo-3. The inhibitory activity has been purified ~2000-fold by normal and reversed-phase HPLC procedures. The inhibitory activity is eluted from the Sephadex G-10 in the range of 350–550 Da, suggesting that the inhibitory factor is a low-molecular-weight substance. The mass spectra analysis shows a number of signals within *m/z* 380–560; however, it is not clear at this moment whether these recordings represent the mass of putative inhibitory factor or irrelevant impurities. The endogenous inhibitory factor of Na/Ca exchange does not resemble the properties (HPLC retention time, mass spectra, amino acid analysis, etc.) of autoinhibitory XIP peptide. The addition of inhibitory factor to muscle strip of guinea pig ventricles induces 2- to 5-fold enhancement of isometric contractions, thereby exhibiting a strong positive inotropic effect. This effect is a dose-dependent phenomenon, which can be reversed by washing the inhibitory factor from the organ bath. Assuming a molecular weight of 350–550 Da, the effective concentrations of putative inhibitor must be $<10^{-6}$ M. Therefore, the present findings demonstrate that the mammalian heart contains a low-molecular-weight factor that can inhibit Na/Ca exchange and enhance the cardiac contractility. © 2000 Academic Press

Key Words: **sodium–calcium exchanger; endogenous inhibitor; positive inotropic effect; calcium homeostasis; cardiac forth; cardiac regulation.**

The cardiac sarcolemma (cell membrane) Na/Ca exchanger protein (NCX1) is a key system for regulating the Ca-extrusion during the action potential (1, 2). Although it is widely accepted that the Na/Ca exchanger is actively involved in excitation-contraction coupling, the underlying regulatory mechanisms are poorly understood. The cardiac Na/Ca exchanger (NCX1) contains an intrinsic inhibitory sequence of 20-amino acids (3), which represents the "calmodulin binding" domain, originally found in the sarcolemma Ca-ATPase (4). The synthetic analog (the XIP peptide) of the autoinhibitory sequence inhibits Na/Ca exchange (5). We found that molluscan "cardioexcitatory (FMRFa-type) peptides" (6) and their synthetic analogs inhibit the mammalian Na/Ca exchange (7–9). Interestingly, no small FMRFa-peptides have been found in mammal (10). All these peptides inhibit Na/Ca exchange from the cytosolic side and they can be successfully used in patch-clamp and vesicular preparations. Unfortunately, the usage of synthetic peptide inhibitors is limited in physiological experiments because of their low permeability to the membrane.

Recent molecular studies clearly show that in animal models of hypertrophied heart and in failing human hearts, the RNA and protein levels of NCX1 increase, while the amount of the SR Ca-ATPase is reduced (11, 12). Although these mechanisms of cardiac remodeling may represent an adaptive response of the cell for avoiding the Ca overload in sarcoplasmic reticulum, these changes could be dangerous for the cell function. For example, the increase of inward depolarizing cur-

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Abbreviations used: 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; 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; PMSF, phenylmethanesulfonyl fluoride; TFA, trifluoroacetic acid; Fluorescamine, 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3-dione.

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[2] To whom correspondence should be addressed. Fax: 972-(0)3-640-9113. E-mail: dhanan@post.tau.ac.il.

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rent by overexpressed Na/Ca exchange may cause a long-lasting slow inward current, which may contribute to the lengthening of the action potential and may create arrhythmogenic ion currents. It has been shown that the peptide blocker, FRCRCFa, inhibits the arrhythmogenic transient inward currents generated by Na/Ca exchange in ventricular myocytes from hypertrophied rat hearts (13).

If the overexpression of the Na/Ca exchanger is so dangerous one may propose that some intrinsic mechanisms might counterbalance this elevated exchanger activity. One possibility is that the Na/Ca exchanger can be down regulated by cytosolic factor(s) the identity of which is not known yet. With this in mind we tested here the hypothesis that the cardiac ventricles contain an endogenous inhibitor that can modulate Na/Ca exchange and muscle contractility. We partially purified a low molecular weight endogenous factor and demonstrated its positive inotropic effect on muscle strip. Although the molecular identity of endogenous inhibitor is not known yet, it does not resemble the properties of any known inhibitor.

MATERIALS AND METHODS

Materials and general procedures. Deoxyribonuclease I (type DN-25, obtained from bovine pancreas), protease inhibitors (PMSF, pepstatin, leupeptin, aprotenin), histidine peptides (carnosine, anserine, carcinine), polyamines (putrescine, spermidine, spermine) and EGTA were purchased from Sigma (St. Louis, MO). Sephadex G-10, G25 and G-50 were from Pharmacia (Uppsala, Sweden). 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). BAPTA and free calcium calibration kit were from Molecular Probes, Inc. (Eugene, OR). Fluo-3 was from Teflabs (Austin, TX). The scintillation cocktail Opti-Fluor for radioactivity counting was from Packard (Groningen, The Netherlands). The XIP peptide was a generous gift of Dr. Philipson, K.D. (UCLA, School of Medicine). All other reagents used in this work were of analytical or reagent (>99.9%) grade purity. The solutions were prepared with deionized water (18 MOhm/cm).

Preparation of sarcolemma vesicles and Na/Ca exchange assays. Large-scale preparations of cardiac sarcolemma vesicles were obtained from fresh calf hearts in the presence of DNase and protease inhibitors (7, 14) according to modified procedure of Jones (22). At −70°C the isolated sarcolemma vesicles retain their Na/Ca exchange activity for 6 months at least. The Na_i-dependent ^{45}Ca-uptake (Na/Ca exchange) and Ca_i-dependent ^{45}Ca-uptake (Ca/Ca exchange) activities were measured by filtration of sarcolemma vesicles on the GF/C filters (23, 24). The reaction time of ^{45}Ca uptake was controlled by automatic injection of quenching solution containing the EGTA buffer (7, 14–17). The standard medium (0.5 ml) for Na/Ca exchange or Ca/Ca exchange assay contained 20 mM Mops/Tris, pH 7.4, 0.25 M sucrose, 35–40 μM $^{45}CaCl_2$ (10^5–10^6 cpm/nmol) and 100–150 μg protein of sarcolemma vesicles. Blanks were measured with 160 mM NaCl in the assay medium and subtracted from the total ^{45}Ca signal (23). Ethanol-free aliquots of heart extracts or purified fractions were lyophilized, dissolved in water and corrected for pH if necessary by concentrated Tris buffer. At all stages of purification procedures the concentrated fractions can be stored at −70°C without any detectable loss of inhibitory activity at lest for 4–5 months. The samples (10–20 μl) of concentrated fractions were added to the assay medium about 2030 min prior the experiment. The ^{45}Ca-uptake assay was initiated by diluting (20- to 50-fold) the Na_i- or Ca_i-loaded vesicles in the assay medium at 37°C.

The reverse mode of Na/Ca exchange (Na_o-dependent Ca efflux) was monitored at 37°C in the SFM-3 stopped-flow machine (BioLogic, Grenoble, France) by using the extravesicular Ca probe, fluo-3 (12,18). In stopped-flow experiments, 50 μl of Ca-loaded vesicles, preequilibrated with the MTS buffer (20 mM Mops/Tris, pH 7.4, and 0.25 M sucrose) were mixed with 50 μl buffer containing 6 μM fluo-3, 20 mM Mops/Tris, pH 7.4, 320 mM NaCl and 0.6 mM BAPTA. Nonspecific signals were recorded and subtracted as described previously (12). The stopped-flow signals were monitored with TC-100/15 cuvette (40 μl volume with 10 mm light path) by fixing a high voltage power supplier at 700–750 V. Fluo-3 was excited at a λ_{ex} of 475 and fluorescence emission was monitored at a λ_{em} of >495 nm (GG-495 long path filter). The SFM-3 stopped-flow system is equipped with three syringe/two mixer device, MOS-200 optical system (mercury–xenon lamp, monochromator with fiber optics and high-voltage supplier) and Hamamatsu R-376 photomultiplier. The stopped-flow conditions (mixing volume, flow rate, etc.) were controlled by the MPF program and the recorded traces were averaged and analyzed with the BioKin 0.14 program, equipped with the Pade-Laplace and Simplex modules (BioLogic, Grenoble, France).

Extraction of endogenous inhibitory activity of Na/Ca exchange from calf ventricles. Fresh calf hearts (1.9 kg), obtained from the closest slaughterhouse, were freed from fat and sliced in small pieces. The frozen tissue was immediately lyophilized. 390 g of dry material was passed through the meat grinder yielding a fine powder. 5 liters of chloroform was added to the powder and then stirred for 15 min at room temperature. This mixture was filtered through the gauze and the filtrate was discarded. The remained pellet was dissolved in 2 liters absolute ethanol and stirred at room temperature for 15 min. The washed pellet was resuspended in 95% ethanol (3l) and homogenized (10 × 30 s) in Polytron PT-3000 (Kinematica AG/Polytron, Lucerne, Switzerland). The homogenized mixture was stirred at room temperature for 1 h and then centrifuged at 12,000*g* for 15 min (GS3 rotor of Sorvall RB5C centrifuge). The pellet was extracted with 95% ethanol once again (as described above) and collected supernatants were evaporated in the hood to remove ethanol. Finally, 500 ml solution was lyophilized to give a final volume of 35–40 ml and all insoluble materials were removed by centrifugation at 50,000*g* for 30 min (SS34 rotor of Sorvall RB5C) following a filtration through the 0.2-μm filter FP30/0.2CA-S (Schleicher & Schuell GmbH, Dassel, Germany). This preparation was used for further purification (see below).

Purification procedures of inhibitory factor. Lyophilized fractions of cardiac ethanol/water extract were dissolved in minimal volume of water and applied on the Sephadex G-10 column (5.5 × 70 cm), preequilibrated with water. The fractions (10 ml) were collected at a flow rate of 2 ml/min by using a Pharmacia GradiFrac chromatography system, equipped with fraction collector, UV-1 optical unit, electrically controlled mixer–injection valves and recorder REC-102. The collected fractions were lyophilized, dissolved in minimal volume of water, and analyzed for peptide content (fluorescamine test) and inhibition of Na/Ca exchange. The HPLC separations were performed by computerized (Millennium software) Waters system, equipped with two M616A pumps, an automatic sample injector (Rheodyne 7725i) and Waters-996 diode array spectrophotometer which can picture the spectra (190–700 nm) of each fraction. The 5-ml injection loop was used in all preparative HPLC runs, while 20-, 50-, or 200-μl injection loops were used for analytical columns. Partially purified material was applied on reverse-phase YMC-Pack C30 (YMC Co., Ltd., Japan) preparative column (20 × 250 mm, 16 μm particle size). The column was washed with water for 30–40 min and then a linear 0–100% acetonitrile gradient applied for 10–20 min. Fractions were collected at a flow rate of 5 ml/min. The fractions containing highest inhibitory activity were collected and loaded on cation-exchange Dowex 50W ×2 column (3.5 × 40 cm) preequilibrated with 0.1% TFA. A pH gradient was generated with HiLoad

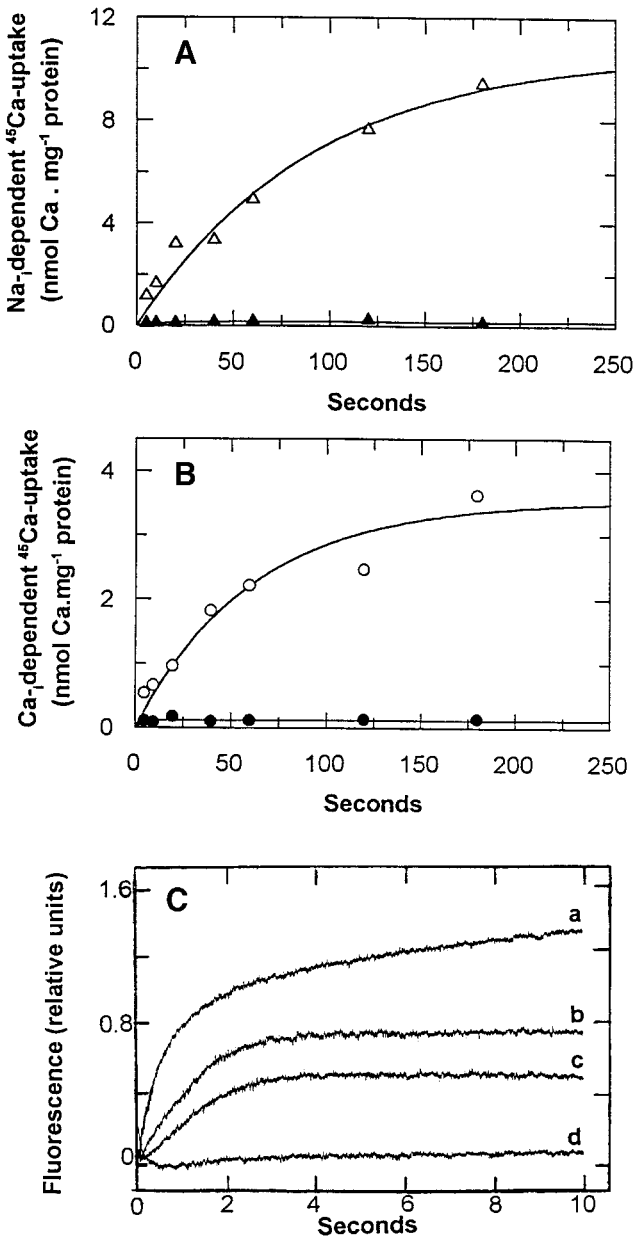

FIG. 1. Effect of cardiac extract on the time course of Na/Ca and Ca/Ca exchange in sarcolemma vesicles. The ethanol/water extracts of calf ventricles were lyophilized and dissolved in water to give about 100- to 300-fold concentration compared to the initial extract (see Materials and Methods). The concentrated cardiac extracts were tested for their capacity to inhibit the forward mode of Na/Ca exchange (A) and its partial reaction the Ca/Ca exchange (B) as well as the reverse mode of Na/Ca exchange (C). The forward mode of Na_i-dependent ^{45}Ca uptake (A) and Ca_i-dependent ^{45}Ca uptake (B) were measured in the absence (△, ○) or presence (▲, ●) of cardiac tissue extracts in the assay medium. Before the experiment 10-μl aliquots (1–2 mg peptide) of concentrated tissue extracts were diluted (50-fold) in the standard assay medium (see Materials and Methods). The Na_i-dependent ^{45}Ca-uptake or Ca_i-dependent ^{45}Ca-uptake reactions were initiated by rapid dilution of Na-loaded (160 mM) or Ca-loaded (0.25 mM) sarcolemma vesicles in the assay medium. At indicated times the ^{45}Ca-uptake reaction was arrested 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). The reverse mode of Na/Ca exchange (Na_o-dependent Ca efflux) was continuously detected by monitoring the fluorescence changes of extravesicular fluo-3 in the stopped-flow machine (C). Equal volumes (50 μl) of Ca-loaded vesicles (syringe A) were mixed with NaCl/BAPTA/fluo-3 buffer (syringe B) to give final concentrations of 160 mM NaCl. Stopped-flow signals of Na_o-dependent Ca efflux were recorded in the absence (trace a) or presence (traces b, c, and d) of endogenous inhibitory factor. Each injection (50 μl) from syringe B included 2 μl (trace b), 5 μl (trace c), or 10 μl (trace d) of concentrated calf heart extracts. Each trace represents an average of at least 5–7 mixings.

pump P-50 (Pharmacia GradiFrac) by mixing 0.1% TFA (pH 2.5) with 10 mM pyridine (pH 7.8). The fractions were lyophilized and analyzed as described above. Active fractions were lyophilized, dissolved in water and analyzed for peptide content and inhibitory activity. The lyophilized material was dissolved in 75% acetonitrile (or acetonitrile) and then loaded on the preparative column (21.5 × 300 mm, 5 μm particle size) of normal phase TSK-gel Amide-80 (TosoHaas, Ltd., Japan), preequilibrated with 75% acetonitrile. The inhibitory activity was eluted at a flow rate of 5 ml/min by decreasing the acetonitrile concentrations from 75 to 35%. The active fractions were lyophilized, dissolved in 50–63% isopropanol. This solution was applied on the same TSK-gel Amide-80 column and the inhibitory activity was eluted under isocratic conditions either with 50 or 75% isopropanol. Retention time of purified preparations of endogenous inhibitory factor were examined on the analytical reversed phase column, μBondapak Waters (4.6 × 250 um, 5 μm particle size). The column was flashed with water at a flow rate of 0.3 ml/min for 10-20 min and then a linear gradient of acetonitrile (0-100%) was applied for 20–30 min. Collected fractions were analyzed for peptide content (20) and inhibitory activity. Lyophilized fractions were dissolved in water and stored at −70°C until use.

Effect of endogenous inhibitory factor on cardiac strip contractility. The guinea pig right ventricles were placed in Krebs–Henseleit solution equilibrated with O_2/CO_2 (95%/5%). Before every experiment the tension of the muscle strip was adjusted to 0.6 g and left for 30 min to equilibrate. To measure contractile force isometrically, about 5-mm-length strips were placed between two platinum-wire electrodes (2 mm apart) embedded in a Lucite holder, and attached to a force transducer (Grass FT03C) by surgical thread (25). The strip was immersed in a 10 ml capacity organ bath connected to an external reservoir with Krebs–Henseleit solution. The temperature (37°C) in the organ bath and reservoir was controlled by thermostat allowing several consecutive washes of muscle strip at constant temperature. The organ bath and reservoir were continuously gassed with a mixture of O_2/CO_2 (95%/5%). The muscle strip was stimulated at 0.5 Hz, 10 V (10 ms pulse duration) providing the optimal conditions for supramaximal stimulation. For testing the inhibitor induced effects on muscle contractility, 10- to 100-μl aliquots of purified preparations were added to the organ bath to give 10^3- to 10^4-fold dilutions of initial stock solutions.

Mass spectra, NMR and amino acid analysis. ^{1}H NMR spectra were recorded on 500 MHz AMX-Bruker NMR machine (responsible person Dr. Shiner, H.) by using D_2O as a solvent. Mass spectra analysis was performed on VG Autospec M250 machine (Fisons Instruments), equipped with digital data system (responsible person Dr. Kugicaro, M., Bar-Ilan University). Mass spectra signals were obtained by using either the positive or negative modes of fast atomic bombardment (FAB) method (26). Amino acid analysis were done by using the amino acid analyzer LC 5000 Biotronic (customer services were provided by Aminolab Ltd., Weizmann Science Park, responsible person Dr. Harduf, Z.).

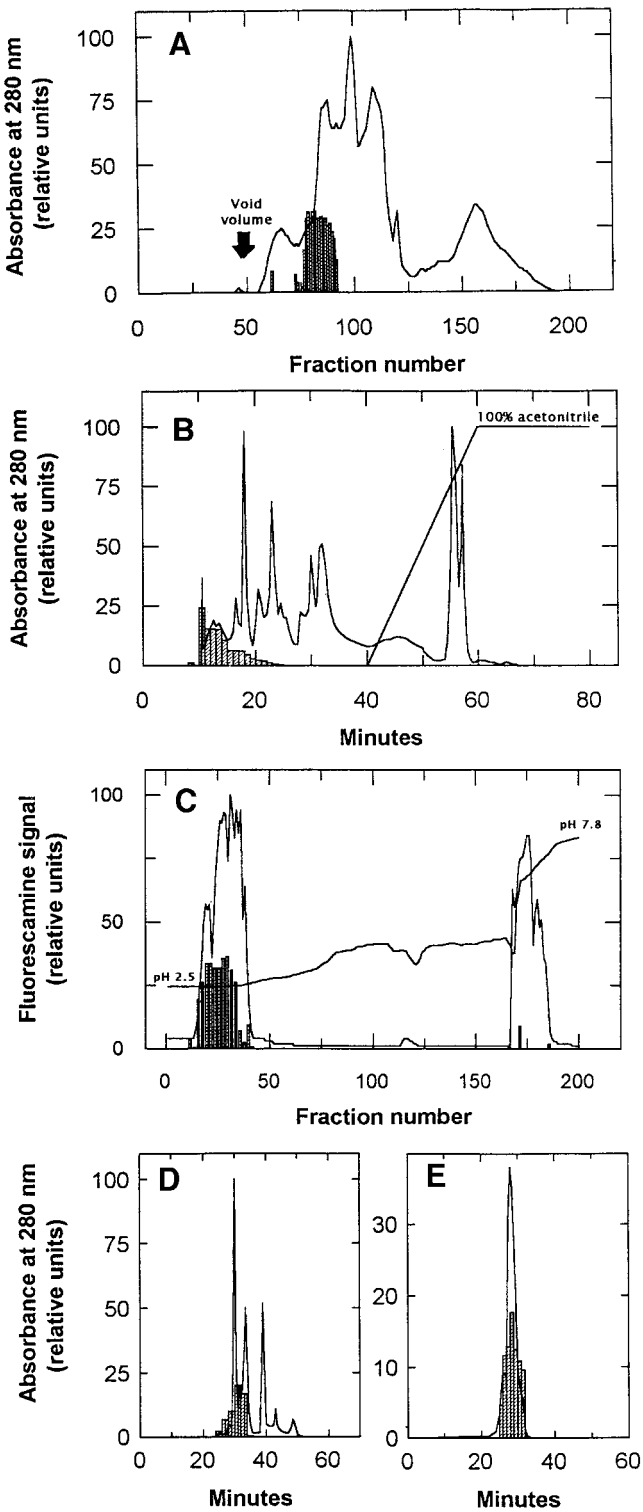

FIG. 2. Chromatographic profiles of Na/Ca exchange inhibition by endogenous factor. Cardiac extracts were successively purified on the Sephadex G-10 (A), YMC-Pack C30 (B), Dowex 50W ×2 (C), and TSK-gel Amide-80 (D and E) columns and the effluents were tested for Na/Ca inhibition and peptide content. In all chromatograms the dashed bars indicate the inhibitory activity of Na/Ca exchange (see Materials and Methods). 30 ml of concentrated cardiac extract was loaded on the Sephadex G-10 column (5.5 × 70 cm) and eluted by water (A). The lyophilized active fractions were dissolved in water (20 ml) and 5-ml portions were applied on the reversed phase YMC-Pack C30 column (20 × 250 mm) in four independent runs. After application of material the column was washed with water (40 min) and then a linear gradient of acetonitrile (0–100%) was applied for 20 min (B). The fractions containing highest inhibitory activity were collected, concentrated to 15 ml and loaded on Dowex 50W ×2 column (3.5 × 40 cm). The pH gradient was generated by mixing 0.1% TFA (pH 2.5) with 10 mM pyridine (pH 7.8) and lyophilized fractions were analyzed for peptide content (fluorescamine assay) and for Na/Ca exchange inhibition (C). Lyophilized active fractions were dissolved in 75% isopropanol and subjected to normal phase chromatography on the TSK-gel Amide-80 column (21.5 × 300 mm). The inhibitory activity was eluted under isocratic conditions with 75% isopropanol (D). Isocratic rechromatography of active fractions was performed on the same column with 50% isopropanol (E). Finally, the lyophilized active fractions were resuspended in water and stored at −70°C until use.

RESULTS AND DISCUSSION

Cardiac extracts, added to the assay medium, inhibit Na/Ca exchange. Lyophilized samples of ethanol/water extracts were dissolved in water and then tested for their effects on Na/Ca and Ca/Ca exchange reactions by using the preparation of isolated sarcolemma vesicles (for details see Materials and Methods). The extracts were added to the assay medium prior the experiment and the Na_i or Ca_i-dependent ^{45}Ca-uptake reactions were initiated by diluting the Na- or Ca-loaded vesicles in the assay medium. At various times (t = 5–180 s) the ^{45}Ca-uptake reactions were arrested by automatic injection of EGTA containing buffer. As can be seen from Fig. 1, the cardiac extracts inhibit the forward mode of Na/Ca exchange (Fig. 1A) as well as its partial reaction the Ca/Ca exchange (Fig. 1B). In parallel set of experiments, the reverse mode of Na/Ca exchange (Na_o-dependent Ca efflux) was continuously monitored in the stopped-flow machine by detecting the fluorescence changes of extravesicular Ca-probe fluo-3 (see Materials and Methods). In this experiments the reaction of Na_o-dependent Ca efflux was initiated by mixing the Ca-loaded vesicles (syringe A) with NaCl/BAPTA buffer (syringe B) containing increasing amounts of extract (traces b, c, and d, Fig. 1C). In control experiments, the cardiac extract was absent in the syringe B (trace a, Fig. 1C). Increasing amounts of crude cardiac extracts inhibit the reverse mode of Na/Ca exchange in a dose-dependent manner (Fig. 1C). These data suggest that the interaction of putative inhibitory factor to its binding site may represent a second-order reaction that may occur within milliseconds.

It is known for a long time that mammalian cardiac tissue contains large quantities of histidine dipeptides and polyamines (28,29). In light of present findings some of these compounds were tested on Na/Ca exchange as related to the endogenous inhibitor in question. These tests show that millimolar concentrations

TABLE 1

Total and Specific Activities of Endogenous Inhibitory Factor at Various Stages of Purification

Fraction	Total peptide content (mg)	Total inhibitory units (inhibitory units)	Specific inhibitory activity (inhibitory units/mg peptide)	Yield (%)
Ethanol extract	2,300	170,000	74	100
Shephadex G-10	73	150,000	2,055	88
C_{30}	51	80,000	1,570	47
Dowex 50W × 2	40	66,000	1,650	39
TSK Amide-80 75% acetonitrile	2	40,000	20,000	24
TSK Amide-80 50% acetonitrile	0.34	40,000	118,000	24

Note. The inhibitory activity was extracted from the calf cardiac tissue and purified as described in the legend to Fig. 2 (see also Materials and Methods). The Na_i-dependent ^{45}Ca uptake was measured *in vitro* by using the preparation of isolated sarcolemma vesicles (for details see Materials and Methods). Standard conditions for Na_i-dependent ^{45}Ca uptake assay are described under Materials and Methods. The peptide content was determined by fluorescamine assay, while for quantitative calibration of fluorescence signals the glycine was used as an external probe. By definition, one inhibitory unit inhibits 1% of Na_i-dependent ^{45}Ca uptake.

of examined histidine peptides (carnosine, anserine, carcinine) and polyamines (putrescine, spermidine, spermine) do not have any significant effect on Na/Ca exchange activity (not shown). Therefore, the tested dipeptides and polyamines cannot be related to currently found endogenous inhibitor of Na/Ca exchange.

Partial purification of endogenous inhibitory factor of Na/Ca exchange. The inhibitory activity of crude cardiac extracts has been purified by 5–6 successive purification steps including the gel filtration, cation-exchange chromatography, reversed phase HPLC and normal phase HPLC (Figs. 2A–2E). From the Sephadex G-10 column the inhibitory activity is eluted in the range of 350–550 Da, suggesting that the endogenous inhibitory factor of Na/Ca exchange might be a small molecule (Fig. 2A). Similar results were obtained by using the Sephadex G-25 and G-50 columns (not shown). From the reversed phase HPLC column (YMC-Pack C30) the inhibitory activity is eluted by water (Fig. 2B), indicating that the putative inhibitory factor is highly soluble in water. Two fluorescamine sensitive picks are eluded from the Dowex 50W ×2 column by pH gradient, while only the first one contains the inhibitory activity (Fig. 2C). The elution of the active pick at pH 2.5 is typical for small neutral or acidic peptide but not for cationic one. The inhibitory activity is retained on the normal phase TSK-gel Amide-80 column and can be eluted by 30–50% organic solvents like acetonitrile, ethanol or isopropanol (not shown). Isocratic chromatography (Fig. 2D) and rechromatography (Fig. 2E) on the TSK-gel Amide-80 column results in a sufficient separation of the inhibitory activity. The eluted inhibitory activity overlays with peptide content profile (detected by fluorescamine), suggesting that the inhibitory factor may be a peptide in nature.

For estimating the yield and specific activity of Na/Ca inhibition at various steps of purification, we defined an arbitrary unit for inhibition (under standard assay conditions one inhibitory unit inhibits the Na_i-dependent ^{45}Ca-uptake by 1%). Table 1 shows that the specific inhibitory activity (number of inhibitory units/mg peptide) is about 1600 times higher in purified preparations compared to crude extracts. The increase of specific inhibitory activity correlates with 6760-fold decrease in total peptide content (Table 1). Moreover, the preparation of purified inhibitory factor shows high yield (20–25% of initial units), meaning that there is no significant loss or irreversible inactivation of the inhibitor during the purification.

HPLC retention time, mass-spectra and amino acid analysis. The inhibitory factor retains its inhibitory activity when exposed (for 10–15 min) to acidic (pH 2–3) or boiling conditions (not shown). The FAB mass spectra analysis of purified preparations shows a number of *m/z* signals in the range of *m/z* 380–560 (Fig.

TABLE 2

Amino Acid Analysis of Endogenous Inhibitory Factor of Na/Ca Exchange

Amino acid	Fractional content by weight (%)	Molar content (nmol)
Aspartic	14.29	0.76
Threonine	1.51	0.09
Serine	3.41	0.23
Glutamic	45.09	2.17
Glycine	22.06	2.08
Alanine	2.89	0.23
Isoleucine	2.59	0.14
Leucine	2.78	0.15
Histidine	3.51	0.16
Lysine	1.86	0.09

Note. The preparation of endogenous inhibitory factor of Na/Ca exchange was obtained as described in the legend to Fig. 2. The samples were hydrolyzed by acidic treatment and then subjected to amino acid analysis according to the established procedures (see Materials and Methods). The samples were injected in the cation-exchange column of LC 5000 Biotronic amino acid analyzer and the amino acids were eluted by pH/salt gradient.

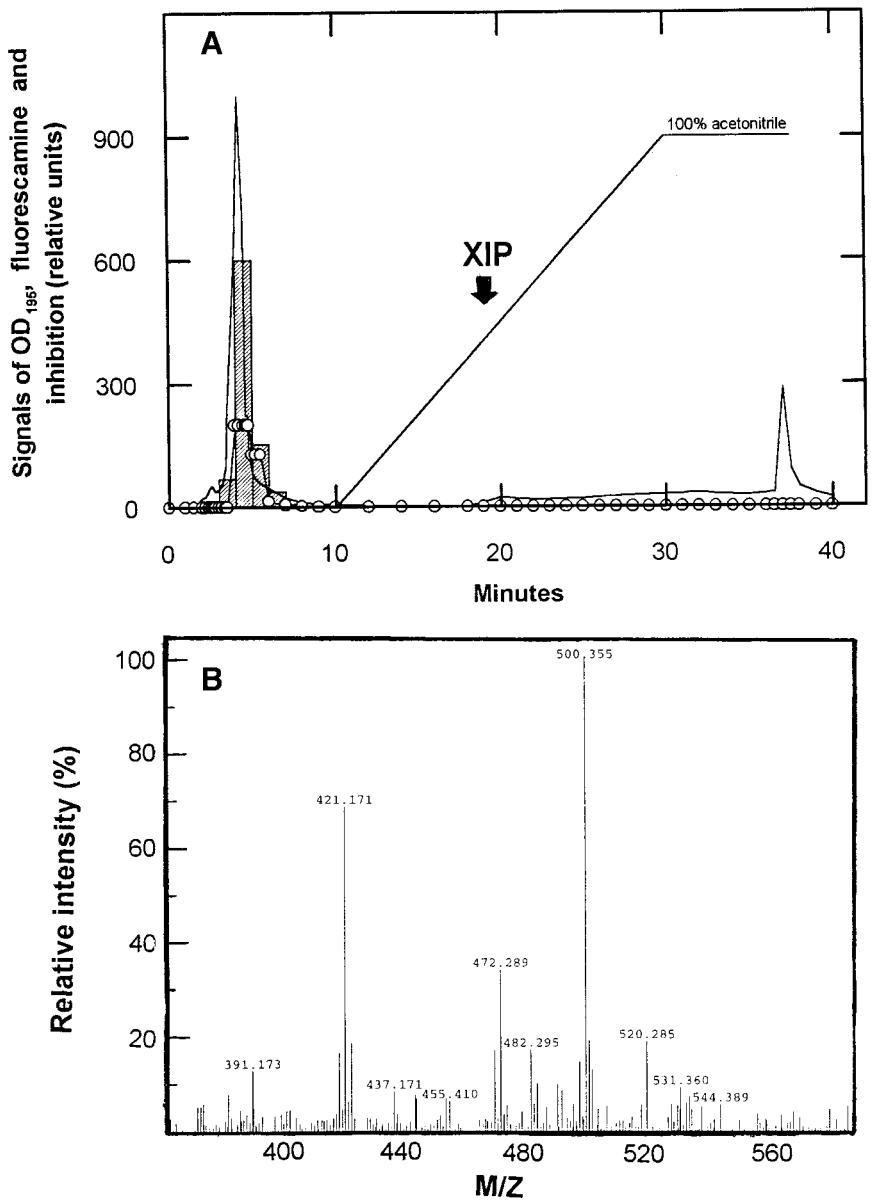

FIG. 3. HPLC-retention time and mass-spectral analysis of purified preparations of endogenous inhibitory factor of Na/Ca exchange. The inhibitory activity of Na/Ca exchange was purified as described in Fig. 2 (see also Materials and Methods) and the HPLC-retention time (A) and mass-spectra profile (B) was determined. The purified preparation of Na/Ca exchange inhibitor or the XIP peptide were loaded on the analytical μBondapak Waters column (4.6 $\times$ 250 mm) in two independent runs. In both cases the column was flashed with water for 10–20 min and then a linear gradient of acetonitrile (0–100%) was applied for 20 min (A). Collected fractions were analyzed for peptide content by fluorescamine (○) and for inhibition of Na/Ca exchange activity (dashed bars). The arrow indicates the elution position of XIP. Purified samples of endogenous inhibitory factor were dissolved in a glycerol matrix solution and positive mode of FAB^+ (fast atomic bombardment) was utilized for recording the mass spectra (B).

3B). It is not clear at this moment whether these data stand for structural properties of the inhibitory factor or they exemplify structural entities of irrelevant substances. A precaution has to be taken that the present preparations of inhibitory factor do not seem represent a homogeneous fraction. For example, ^{1}H NMR analysis shows that the purified preparations contain detectable amounts of unidentified mono and/or disaccharides (not shown), so their contribution to inhibitory activity cannot be excluded at this moment. Unfortunately, even most advanced HPLC techniques (30–32) cannot guarantee at this moment a complete separation of water-soluble (low molecular weight) peptides from simple sugars. A combination of chemical modification and mass-spectral analysis is perhaps required to overcome this problem.

In light of present findings one may suggest that endogenous proteolytic enzymes may cleave the auto-

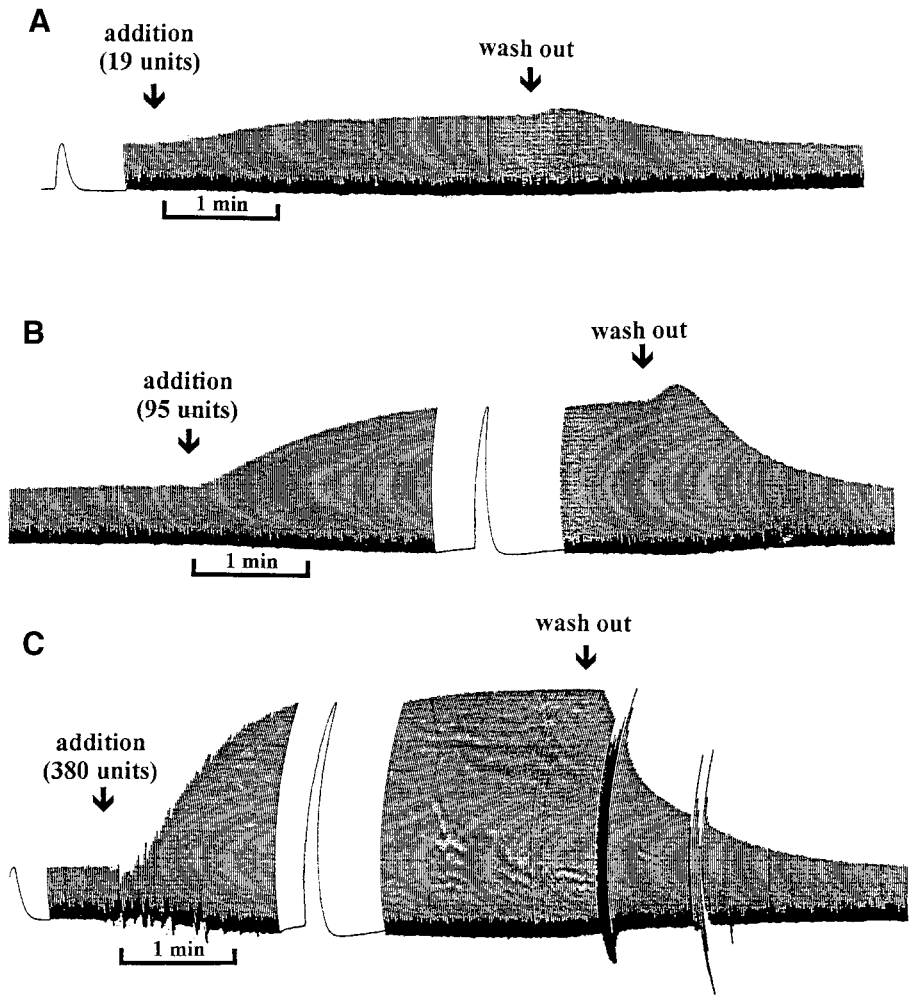

FIG. 4. Positive inotropic effect induced by addition of endogenous inhibitory factor of Na/Ca exchange to cardiac muscle strip. The isolated strip of guinea pig right ventricle was placed in temperature-controlled organ bath (37°C). The isometric contractile force was measured by electric excitation (0.5 Hz) of muscle strip (for details see Materials and Methods). At indicated times, the preparation of purified endogenous inhibitory factor (see Fig. 2) was directly diluted 10^3- to 10^4-fold in the organ bath to give 19 (A), 95 (B) or 380 (C) inhibitory units in the organ bath (10 ml). By definition, one inhibitory unit inhibits 1% of Na_i-dependent ^{45}Ca uptake in sarcolemma vesicles, determined under standard assay conditions (see Materials and Methods). The inhibitory factor was washed from the organ bath at indicated times (shown by arrows) by rapid injection of fresh ringer solution at constant temperature.

inhibitory XIP domain of NCX1 yielding a soluble inhibitory peptide (MW 2557 Da) or its derivative. Therefore, one should examine a possible relation of found endogenous inhibitor with the XIP peptide. First, the endogenous inhibitory activity is eluted by water from the analytical C_{18} column showing a retention time of 4.3 min, while the XIP peptide is eluted with 38–40% acetonitrile from the same column (Fig. 3A). Second, the computer simulated FAB profiles (26, 27) of XIP (and its possible derivatives) do not fit at all with the observed FAB signals of endogenous inhibitor (Fig. 3B). Finally, the amino acid analysis shows an enrichment of purified preparations with Asp, Glu and Gly (Table 2), but not with Lys, Val, Arg and Tyr as one might expect for the XIP sequence. Therefore, at this stage of available analysis the endogenous inhibitor does not resemble the physicochemical properties of the XIP peptide. Moreover, the properties of endogenous inhibitor also differ from the molluscan FMRFa-type peptides (and their synthetic analogs) because the former peptides contain more then one positively charged amino acid plus aromatic residue (6–8). The endogenous inhibitor of Na/Ca exchange seems to be a very hydrophilic molecule, so it cannot resemble the properties of 'steroid-type' endogenous inhibitors of Na/K ATPase either.

Endogenous inhibitory factor can enhance the contractility of ventricle muscle strip. The effects of purified preparations of endogenous inhibitor were tested on isometric contractions by using the isolated muscle strips obtained from guinea pig right ventricles. This

preparation is very useful for testing the physiological effects, because the added substances can rather easily penetrate through the tissue via gap junctions. In these experiments the ventricle muscle strip (immersed with ringer solution in the organ path) was excited with short electric pulses at constant frequency (for details see Materials and Methods). The purified preparations (2 inhibitory units/μl) of inhibitor were diluted (50- to 1000-fold) in the ringer solution to give 19–380 total inhibitory units in the organ bath (Fig. 4). The addition of 19 units to muscle strip results in about 2-fold enhancement of contraction amplitude within 2-3 min (Fig. 4A). Moreover, the muscle contractility was increased 3-fold and 5-fold when 95 (Fig. 4B) and 380 (Fig. 4C) units were added to the organ bath, respectively. After the addition of inhibitor to muscle strip it takes about 2–3 min to rich maximal levels of positive inotropic effect (Fig. 4), suggesting that the substance might overcome some diffusion barrier before it exposes to the interacting site. Assuming a molecular weight of 350–550 Da, one may calculate that submicromolar (or even lower) concentrations of putative inhibitor might be effective for generating the positive inotropic effect. By washing the inhibitory factor from the ringer medium the positive inotropic effects can be expunged to basal levels within few minutes (Fig. 4). Therefore, the present data suggest that the inhibitor induced positive inotropic effect is a dose dependent phenomenon that can be reversed in time. The inhibitor-induced effects were highly reproducible in experiments with seven guinea pigs and three different preparations of purified inhibitor.

In summary, this work shows for the first time that cardiac ventricle extracts contain a low molecular weight factor, which has a capacity to inhibit the Na/Ca exchange activity. The preparation of partially purified endogenous inhibitor is also able to enhance the cardiac contractility, thereby exhibiting a strong positive inotropic effect on mammalian cardiac tissue. Molecular identification of this endogenous inhibitor of Na/Ca exchange is currently under investigation in our laboratory.

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