Biochimica et Biophysica Acta, 1068 (1991) 97–104

ADONIS 00052/3091003342

BBAMEM 75355

Sodium-calcium exchange in membrane vesicles from aortic myocytes: Stimulation by endogenous proteolysis masks inactivation during vesicle preparation

Rong-Ming Lyu [1], John P. Reeves [2] and Jeffrey Bingham Smith [1]

[1] *Department of Pharmacology, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, AL (U.S.A.) and* [2] *Roche Research Center, Roche Institute of Molecular Biology, Nutley, NJ (U.S.A.)*

(Received 27 February 1991)
(Revised manuscript received 20 May 1991)

Key words: Smooth muscle; Cardiac muscle; Calcium transport; ATP; Proteinase

Plasma membrane vesicles were purified from rat aortic myocytes by centrifugation in a discontinuous sucrose gradient. Vesicles were prepared in the presence or absence of five proteinase inhibitors (aprotinin, benzamidine, leupeptin, pepstatin A and phenylmethylsulfonyl fluoride). The proteinase inhibitors decreased the V_{max} by 3.4-fold and had no effect on the K_m for Ca^{2+} of Na^+ gradient-dependent $^{45}Ca^{2+}$ influx. The proteinase inhibitors had no direct effect on exchange activity, and they had no effect on membrane purity as indicated by 5'-nucleotidase activity. Removing the proteinase inhibitors or adding trypsin or chymotrypsin increased exchange activity approx. 2-fold. The V_{max} of exchange activity in intact aortic myocytes is approx. 10-fold higher than the V_{max} in plasma membrane vesicles prepared in the presence of proteinase inhibitors. Exchange activity in plasma membrane vesicles is only a sixtieth of the expected value, because the vesicles have approx. 7-fold higher 5'-nucleotidase activity and approx. 6-fold higher specific exchange activity than the crude homogenate. The large loss of exchange activity may be caused by a change in a regulatory domain of the exchanger because endogenous proteolysis restores some of the activity lost during vesicle preparation.

Introduction

An Na^+-Ca^{2+} exchange mechanism is plentiful in the plasma membrane of certain cell types including nerve [1–3], cardiac muscle [4,5] and certain renal epithelial [6–9] and smooth muscle cells [10–13]. Metabolic energy does not directly fuel Na^+-Ca^{2+} exchange because it is active in dialyzed squid axons [14,15] and sarcolemmal vesicles [5] in the absence of a source of metabolic energy. ATP, however, activates Na^+-Ca^{2+} exchange in squid axons [14,15] and giant sarcolemmal patches from cardiac cells [16]. Protein phosphorylation appears to cause the activation of the exchanger because non-hydrolyzable ATP analogues had no effect on exchange activity [16–18]. Recently, we [19] reported that Na^+-Ca^{2+} exchange in aortic myocytes depends on metabolic energy. Three different classes of mitochondrial poisons rapidly decreased cell ATP and strongly inhibited exchange activity [19]. Glucose prevented the poisons from decreasing ATP and exchange activity indicating that the poisons do not directly affect the exchanger. Moreover, the time-courses of the disappearance and restoration of exchange activity closely parallel those of ATP depletion and repletion [19]. Metabolic energy increased the V_{max} of exchange in the smooth muscle cells [19] rather than increasing the apparent affinities of the exchanger for Na^+ and Ca^{2+} as shown in dialyzed squid axons [14,15,18].

The initial goal of the present study was to use plasma membrane vesicles from aortic myocytes to further clarify the modulation of Na^+-Ca^{2+} exchange by metabolic energy. We have found that plasma membrane vesicles have high exchange activity providing

Abbreviations: DPCC, diphenylcarbamyl chloride; Mops, (3-[*N*-morpholino]propanesulfonic acid; KMops, potassium chloride-Mops solution; NaMops, sodium chloride-Mops solution; P1, SN1, SN2P, F1, and F2 are pellets and supernatants as defined in Materials and Methods; TLCK, L-(tosylamido-2-phenyl)ethylchloromethyl ketone.

Correspondence: J.B. Smith, Department of Pharmacology, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, AL 35294, U.S.A.

the vesicles are prepared in the absence of proteinase inhibitors. The stimulation of exchange by endogenous proteolysis partially masks the large losses of activity that occur during vesicle preparation. Exchange activity in the vesicles appears to be deregulated with respect to metabolic energy.

Materials and Methods

Plasma membrane purification

Smooth muscle cells were isolated from rat aorta and grown in Medium 199 containing 10% (v/v) fetal bovine serum (Hyclone laboratories, Logan, UT) as previously described [10,20]. The cultures (10 cm diameter) were used between passage 5 and 15. They were rinsed three times with ice-cold saline containing 10 mM Hepes which was adjusted to pH 7.4 with Tris. The cells were removed from the culture surface with a squeegee, collected by centrifugation, and suspended with an ice-cold buffer which contained 160 mM NaCl and 20 mM Mops adjusted to pH 7.4 with Tris (NaMops). Proteinase inhibitors (4 μg/ml pepstatin A, 4 μg/ml leupeptin hemisulfate, 40 KIU/ml aprotinin (10000 KIU/mg solid), 0.8 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine-HCl) were present in the NaMops and all solutions used for membrane purification unless otherwise indicated. The cell suspension was incubated in a Parr Nitrogen Cavitation Chamber at 500 lb/inch^2 for 10 min before slowly releasing the broken cells from the chamber. The homogenate was centrifuged at $900 \times g$ for 5 min. The pellet of unbroken cells was homogenized and centrifuged again. The supernatants were combined and centrifuged at $1700 \times g$ for 5 min to produce a pellet rich in nuclei (P1) and a supernatant (SN1). After adding sucrose (1 ml of 40% (w/w) in 20 mM Mops/Tris (pH 7.4) per 4 ml of SN1), mitochondria were removed by centrifugation at $7000 \times g$ for 5 min, and the supernatant was centrifuged at $125000 \times g$ for 45 min to produce a microsomal pellet (SN2P).

Microsomal membranes were fractionated on a discontinuous sucrose gradient as previously described [13]. The microsomal pellet was suspended with 4 ml 8% sucrose and layered on two gradients composed of 5 ml 33% sucrose on 5 ml of 40% sucrose and centrifuged at $125000 \times g$ for 90 min in a swinging bucket rotor (SW 41, Beckman Instruments, Fullerton, CA). The sucrose solutions contained 20 mM Mops adjusted to pH 7.4 with Tris. The membranes at the interfaces between 8 and 33% sucrose (F1) and between 33 and 40% sucrose (F2) were collected ($\sim$ 2 ml each), diluted at least 3-fold with NaMops, and sedimented by centrifugation at $125000 \times g$ for 45 min. The pellets were suspended with NaMops at 3–10 mg protein/ml. The pellet from the sucrose gradient was suspended in 0.25 ml of NaMops and assayed for marker enzymes. Protein was measured by the method of Lowry et al. [21] with bovine serum albumin as the standard (Bio-Rad Laboratories, Richmond, CA).

Assays of marker enzymes

The plasma membrane marker, 5'-nucleotidase, was assayed by the method of Heppel and Hilmoe [22]. The vesicles were frozen slowly and stored at -20°C prior to the assay. Detergent was not used because the presence of Triton X-100 or digitonin in the assay solution had little or no effect on the 5'-nucleotidase activity as indicated by the following result. SN2P vesicles were incubated with 0.06% (v/v) Triton X-100 or 0.025% (w/v) digitonin for 5 min before starting the assay by adding AMP. The reaction was stopped after 15 min by the addition of trichloroacetic acid, and inorganic phosphorus was measured with the Elon-Molybdate method [23]. 5'-Nucleotidase activity in the presence of Triton X-100 or digitonin was $108 \pm 2\%$ or $102 \pm 2\%$ of the value in the absence of detergent ($n = 6$ experiments), respectively. The mitochondrial marker, cytochrome *c* oxidase, was assayed by the method of Cooperstein and Lazarow [24]. The reaction mixture contained 71.1 μM horse heart cytochrome *c* (Type VI), reduced by $Na_2S_2O_4$, in 50 mM potassium phosphate buffer (pH 7.4). The decrease in absorbance at 550 nm was monitored for 4 min after starting the reaction by adding enzyme. Assays were done in duplicate or triplicate. Total and specific enzyme activities were monitored in each step of membrane purification.

Na^+-Ca^{2+} exchange assay

Sarcolemmal vesicles (F1 fraction) were suspended with 0.2 ml NaMops, and incubated on ice for 30 min before assaying Na^+-Ca^{2+} exchange as Na^+ gradient-dependent $^{45}Ca^{2+}$ uptake as described by Reeves [25]. Briefly, 2 μl of vesicles was placed on the side of a polystyrene tube (85 × 13 mm, Sarstedt) which contained 0.1 ml of assay buffer: NaMops or KMops (160 mM KCl instead of NaCl) adjusted to pH 7.4 at 37°C with Tris, and 5–15 μCi/ml $^{45}Ca^{2+}$ (10 μM total Ca^{2+} unless indicated otherwise). Uptake was started by vortexing and stopped by adding 4 ml of ice-cold quench solution: 200 mM KCl, 5 mM Mops, 0.1 mM EGTA and Tris to pH 7.4 at 2°C. Vesicles were collected on microfiber filters (GF/A, Whatman International, Maidstone, U.K.), which were soaked in 0.3% (w/v) polyethylenimine, and washed twice with the quench solution. A 'zero-time' blank was determined by simultaneously mixing the vesicles with quench and assay solution. The 'zero time' values of $^{45}Ca^{2+}$ uptake were always less than 5% of steady state $^{45}Ca^{2+}$ uptake (1–5 min) in NaMops. Na^+-Ca^{2+} exchange activity was taken as the difference between $^{45}Ca^{2+}$ uptake (nmol/mg protein) in KMops and NaMops at 15 s. Exchange

activity increased linearly with time for at least 30 s (Fig. 1).

To determine the dependence of Na^+-Ca^{2+} exchange on external Ca^{2+}, the assay solutions were passed through a Chelex-100 column (0.9 × 22 cm). Chelex-100 was prepared in the Na^+ or K^+ form by the method of Blinks et al. [26] and used for NaMops or KMops, respectively. A 15 s incubation with $^{45}Ca^{2+}$ in NaMops or KMops was used to determine exchange activity. The assay solutions contained 0.75 μCi $^{45}Ca^{2+}$ when total Ca^{2+} was 5 μM, 1.5 μCi for 10, 20 and 50 μM Ca^{2+}, and 6.0 μCi for 75, 100, 200, 400 and 600 μM Ca^{2+}. The kinetic parameters (mean ± S.E.) were obtained from the data in Fig. 3 by non-linear regression fitting to the Michaelis-Menten equation with Enzfitter (Elsevier-BIOSOFT, Cambridge, U.K.). A statistical comparison of the kinetic parameters was done by the method of Cleland [27]. All assays of Na^+-Ca^{2+} exchange activity were done at least in triplicate.

$^{22}Na^+$ efflux

Vesicles (SN2P) were prepared from 12 cultures (10 cm diameter) in the presence or absence of the proteinase inhibitors as described above except a Dounce homogenizer with a tightly-fitting pestle (50 strokes) was used instead of N_2 cavitation. The vesicles (150 μl, ~ 0.75 mg protein) were suspended in NaMops incubated on ice for approx. 16 h with 50 μl $^{22}Na^+$ (1 mCi/ml water). Efflux was initiated by mixing 8 μl of vesicles with 100 μl of KMops containing 10 μM $CaCl_2$. At the indicated times the vesicles were diluted with 4.5 ml of ice-cold 0.1 M $MgCl_2$ containing 10 mM Hepes/Tris (pH 7.4), filtered and rinsed twice with 4.5 ml of the same solution. A 'no vesicle' blank obtained by filtering the same amount of $^{22}Na^+$ in the same way as for vesicles was subtracted to obtain vesicular $^{22}Na^+$. The 'zero time' value of vesicular $^{22}Na^+$ was determined by simultaneously mixing the vesicles with the rinse and incubation solutions. Assays were done in triplicate.

Materials

Proteinase inhibitors, DPCC-treated trypsin, TLCK-treated chymotrypsin, cytochrome *c*, Chelex-100 and polyethylenimine were from Sigma (St. Louis, MO). $^{45}CaCl_2$ (16–34 Ci/g) and $^{22}NaCl$ (840 Ci/g) was from DuPont, New England Nuclear Research Products (Boston, MA).

Results

Plasma membrane purification and general properties of Na^+-Ca^{2+} exchange

We have verified that centrifugation on the discontinuous sucrose gradient devised by Matlib [28] as modified by Slaughter et al. [13] considerably improved the specific activities of 5'-nucleotidase and Na^+-Ca^{2+} exchange and strongly decreased mitochondrial contamination as reported previously for aortic tissue [13]. The specific activities of cytochrome oxidase and 5'-nucleotidase in the crude homogenate (SN1) were 135 ± 18 nmol/min per mg protein and 28 ± 8 nmol/min per mg protein (mean ± S.E., $n = 3$ experiments). The specific activity of 5'-nucleotidase increased about 7-fold to 206 ± 24 nmol/min per mg protein in the F1 fraction from the sucrose gradient ($n = 3$ experiments). This fraction contained 56.1% of total 5'-nucleotidase activity. The specific activity of Na^+-Ca^{2+} exchange was 5.8-fold higher in this fraction compared to the crude membranes. The specific activity of cytochrome oxidase was 75.4% lower in F1 compared to SN1, and F1 contained only 1.1% of total cytochrome oxidase activity.

Ca^{2+} uptake was much faster and reached about a 10-fold higher steady state level in KMops compared to NaMops (Fig. 1). Furthermore, high intravesicular Na^+ was essential for producing rapid $^{45}Ca^{2+}$ uptake. Vesicles prepared in KMops showed only about a 50% increase in the rate and extent of $^{45}Ca^{2+}$ uptake in KMops compared to NaMops [38]. Additionally, diluting the K^+ vesicles with NaMops to load them with Na^+ largely restored the high rate of $^{45}Ca^{2+}$ uptake in KMops [38].

Valinomycin stimulates Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles [29–31] and to a lesser extent in membrane vesicles from arterial tissue [11–13]. Vali-

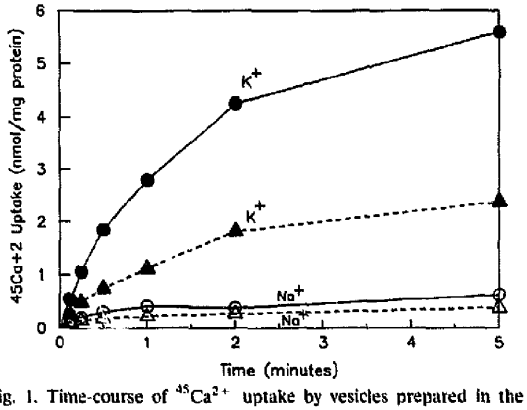

Fig. 1. Time-course of $^{45}Ca^{2+}$ uptake by vesicles prepared in the presence (dotted lines) or absence of proteinase inhibitors (solid lines). 3 days after seeding the cultures half of them were used to prepare plasma membranes (F1) in the presence (triangles, dotted lines) and half were used to prepare plasma membranes in the absence of proteinase inhibitors (circles, solid lines) as described in Materials and Methods. $^{45}Ca^{2+}$ uptake was measured with 10 μM $^{45}Ca^{2+}$ in NaMops (unfilled markers) or KMops (filled markers). Values are means of triplicates. S.E. values are all smaller than the diameters of the markers.

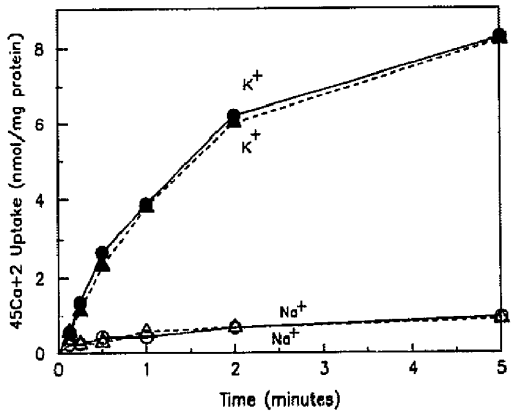

Fig. 2. Lack of an effect of proteinase inhibitors on $^{45}Ca^{2+}$ uptake by plasma membrane vesicles prepared in the absence of the inhibitors. F1 vesicles were prepared in the absence of the inhibitors and incubated for 2 h on ice with (triangles, dotted lines) or without (circles, solid lines) 4 μg/ml pepstatin A, 4 μg/ml leupeptin, 40 KIU/ml aprotinin, 0.8 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine before assaying $^{45}Ca^{2+}$ uptake. Uptake was assayed in NaMops (unfilled markers) or KMops (filled markers) at 10 μM Ca^{2+}. Three additional experiments confirmed that proteinase inhibitors had no effect on exchange activity of vesicles prepared without them. Values are means of triplicates. S.E. values are all smaller than the diameters of the markers.

nomycin apparently stimulates exchange by collapsing the membrane potential produced by the efflux of 3 Na^+ per Ca^{2+} taken up by the vesicles. Because valinomycin is hydrophobic and does not remain in solution for long [25], a 2 mM solution was made with dimethyl sulfoxide and dilutions were used within a few min. Valinomycin (0.5–2 μM) had no effect on $^{45}Ca^{2+}$ uptake by vesicles (F1 or SN2P) from aortic myocytes in the K^+ or Na^+ assay solutions (Lyu, unpublished data). Therefore, valinomycin was not routinely used for assaying exchange activity. Although the exchanger in smooth muscle appears to be electrogenic [32,33], high basal permeability to Na^+, K^+, and/or Cl^- relative to Na^+-Ca^{2+} exchange activity may prevent the polarization of the membrane during the exchange assay (see $^{22}Na^+$ efflux data below).

Preparing vesicles in the presence of proteinase inhibitors decreases Na^+-Ca^{2+} exchange

Preparing the vesicles in the presence of five proteinase inhibitors (4 μg/ml pepstatin A, 4 μg/ml leupeptin, 40 KIU/ml aprotinin, 0.8 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine) strongly decreased the rate of $^{45}Ca^{2+}$ uptake in KMops and had no effect on uptake in NaMops (Fig. 1). These data indicate that the vesicles prepared in the absence of the proteinase inhibitors have much higher Na^+-Ca^{2+} exchange activity than those prepared in the presence of the inhibitors. The proteinase inhibitors may prevent or decrease the extent of the activation of the exchanger by endogenous proteolysis. Alternatively, the proteinase inhibitors may directly affect exchange activity. Adding the inhibitors to vesicles prepared without them had no effect on $^{45}Ca^{2+}$ uptake in KMops or NaMops (Fig. 2) indicating that the inhibitors have no direct effect on Na^+-Ca^{2+} exchange activity. Therefore, it is likely that the inhibitors decrease exchange activity by preventing or decreasing the extent of the activation of the exchanger by endogenous proteolysis.

Next we examined the Ca^{2+} concentration dependence of exchange activity in vesicles prepared with or without the proteinase inhibitors. Preparing F1 vesicles in the presence of the proteinase inhibitors decreased the V_{max} by 3.4-fold (Fig. 3). The V_{max} of exchange was 3.56 ± 0.16 and 1.05 ± 0.05 nmol/15 s per mg ($P < 0.05$) for F1 vesicles prepared in the absence or presence of the proteinase inhibitors, respectively. In contrast to the striking effect on the V_{max}, preparing F1 vesicles in the presence of the inhibitors had no effect on the apparent K_m of the exchanger for Ca^{2+}. The K_m was 33 ± 5 and 24 ± 4 μM ($P > 0.05$) for vesicles

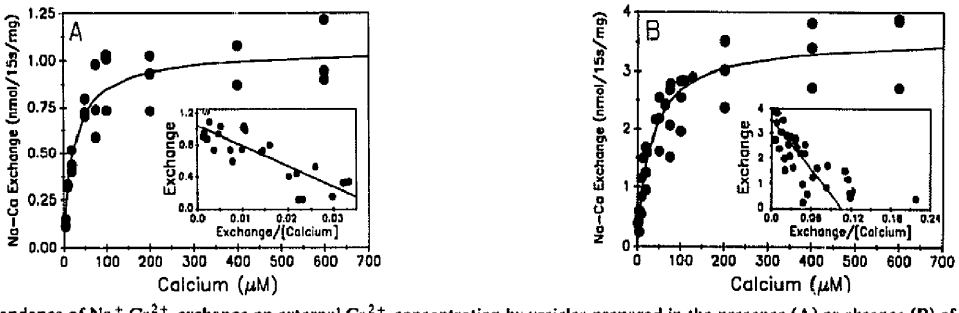

Fig. 3. Dependence of Na^+-Ca^{2+} exchange on external Ca^{2+} concentration by vesicles prepared in the presence (A) or absence (B) of proteinase inhibitors. $^{45}Ca^{2+}$ uptake in NaMops was subtracted from uptake in KMops to obtain exchange activity. Each data point is the mean of triplicates. The data are from four plasma (F1) membrane preparations done in the presence of the proteinase inhibitors and four in their absence. The curves were obtained by non-linear least squares fitting to the Michaelis-Menten equation. Insets: Eadie-Hofstee plots of the data. Values are means of triplicates. S.E. values are all smaller than the diameters of the markers.

prepared in the absence or presence of the inhibitors (Fig. 3).

We also compared specific exchange and 5'-nucleotidase activities of SN2P vesicles to find out if the proteinase inhibitors affected the purity of the vesicles. SN2P vesicles were prepared by Dounce homogenization in the presence and absence of the inhibitors as indicated in Materials and Methods. Both vesicle preparations were done at the same time from cultures that were seeded on the same day. 5'-Nucleotidase activity was 94 ± 14 and 80 ± 6 nmol/min per mg ($n = 3$ experiments) for vesicles prepared in the presence or absence of the inhibitors, respectively. Because there was no significant difference ($P > 0.05$) in 5'-nucleotidase activity, preparing the vesicles in the presence of the proteinase inhibitors probably has no effect on the purity of the vesicles. Exchange activity, measured at 24 μM Ca^{2+}, was 2.2-fold higher in SN2P vesicles prepared in the absence of the inhibitors (0.54 ± 0.07 versus 0.25 ± 0.02 nmol $^{45}Ca^{2+}/15$ s per mg ($n = 8$ experiments). The somewhat smaller effect of the proteinase inhibitors on the exchange activity of SN2P compared to the F1 vesicles may reflect a small change in the Ca^{2+} concentration dependence of exchange as well as a greater degree of endogenous proteolysis with F1 vesicles, which take considerably more time to prepare than SN2P vesicles. These data indicate that differential purification does not account for the increased exchange activity of vesicles prepared in the absence of the proteinase inhibitors.

Rapid loss of vesicular $^{22}Na^+$

Including the proteinase inhibitors in the solutions used to purify plasma membranes decreased the apparent steady state level of $^{45}Ca^{2+}$ uptake (Fig. 1). Rapid dissipation of the Na^+ gradient may account for the lower steady state level of $^{45}Ca^{2+}$ in the vesicles prepared in the presence of the proteinase inhibitors. $^{22}Na^+$ efflux measurements from SN2P vesicles showed that there was a substantial loss of vesicular Na^+ during the first min of the exchange assay (Fig. 4). About 50% of vesicular $^{22}Na^+$ was lost during the first minute of incubation in KMops containing 10 μM $CaCl_2$ (Fig. 4). Additionally, the time-course of $^{22}Na^+$ efflux was the same for vesicles prepared with or without the proteinase inhibitors (Fig. 4), although the vesicles prepared without the inhibitors had 2.2-fold higher exchange activity under these conditions as indicated above. The lower steady state level of $^{45}Ca^{2+}$ uptake by vesicles prepared with the proteinase inhibitors may be caused by the dissipation of the Na^+ gradient and the lower initial exchange activity of the vesicles prepared with the inhibitors compared to those prepared without them (Fig. 1). The rapid dissipation of the Na^+ gradient during the assay also may dissipate any membrane charge produced by the exchanger and thereby account for the failure of valinomycin to stimulate Na^+-Ca^{2+} exchange.

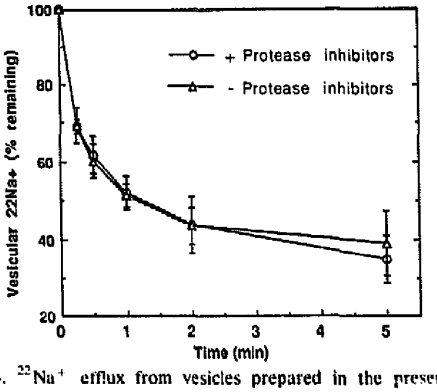

Fig. 4. $^{22}Na^+$ efflux from vesicles prepared in the presence or absence of proteinase inhibitors. Values are mean ± S.E. for three experiments on SN2P vesicles prepared with the inhibitors and two experiments without inhibitors which were done by a paired design at the same time as the last two experiments with inhibitors.

Removing the proteinase inhibitors and adding a proteinase increases Na^+-Ca^{2+} exchange

Sarcolemmal vesicles were prepared in the presence of the proteinase inhibitors. The F1 fraction was di-

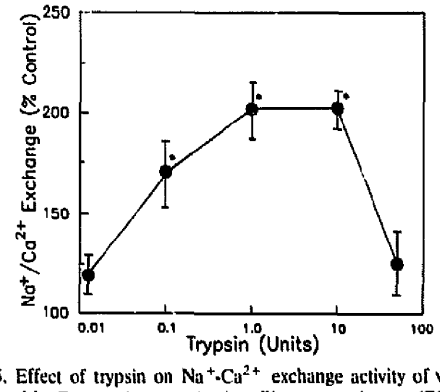

Fig. 5. Effect of trypsin on Na^+-Ca^{2+} exchange activity of vesicles prepared by Dounce homogenization. Plasma membranes (F1) were prepared in the presence of the proteinase inhibitors as indicated in Materials and Methods except the cells were homogenized by 50 strokes of a tightly-fitting pestle using a Dounce homogenizer instead of N_2 cavitation. 40 μl of vesicles (~0.120 mg protein) was incubated for 20 min at 37°C with 5 μl of NaMops (control) or 5 μl of DPCC-treated trypsin to give the indicated number of NF units. Then 5 μl of soybean trypsin inhibitor (3 mg/ml, Type I-S) was added, and the vesicles were incubated on ice for 40 min before assaying exchange activity. Because the specific activity of the trypsin was 2570 NF units/mg protein, incubation with 1 unit of trypsin corresponds to a 1:324 ratio of trypsin protein to membrane protein. The exchange activity of vesicles incubated without trypsin was 0.375 nmol $^{45}Ca^{2+}$/mg protein 15 s. Values are mean ± S.E. ($n = 5$). An asterisk denotes a statistically significant difference ($P < 0.05$) by Student's t-test.

TABLE I

Effects of removing the proteinase inhibitors or adding trypsin or chymotrypsin on Na^+-Ca^{2+} exchange

Plasma membrane vesicles (F1) were prepared in the presence of the proteinase inhibitors and diluted 4-fold with NaMops with (A) or without (B) the inhibitors. The vesicles were sedimented (125000 × *g* for 45 min) and suspended with 0.2 ml NaMops without the inhibitors. 40 μl of vesicles (~ 80 μg protein) was incubated for 20 min at 37°C with 5 μl of NaMops ('None') or 5 μl DPCC-treated trypsin (2570 NF units/mg protein) or TLCK-treated chymotrypsin (50 units/mg protein, Type VII). Concentrated (10×) solutions of the proteinases were prepared in NaMops. Finally, 5 μl of a mixture of the five proteinase inhibitors at 10× the concentration indicated in Materials and Methods was added to the vesicles which were then incubated for 40 min on ice before assaying exchange activity. $^{45}Ca^{2+}$ uptake was measured after a 15 s incubation in NaMops and KMops at 10 μM $^{45}Ca^{2+}$. *n* equals the number of experiments; each was done in triplicate. Values are mean ± S.E. An asterisk indicates $P < 0.05$ compared to the controls (no added proteinase) by Student's *t*-test.

Proteinase added	Na^+-Ca^{2+} exchange (nmol $^{45}Ca^{2+}$/15 s per mg)	*n*
(A) Vesicles diluted with inhibitors		
none	0.26 ± 0.08	6
trypsin, 0.5 NF units	0.41 ± 0.04 *	3
trypsin, 5 NF units	0.47 ± 0.05 *	6
chymotrypsin, 1 unit	0.45 ± 0.12 *	3
(B) Vesicles diluted without inhibitors		
none	0.51 ± 0.09	4
trypsin, 5 NF units	0.73 ± 0.07 *	4

luted 4-fold with ice-cold NaMops with inhibitors, and the vesicles were collected by centrifugation and resuspended in ice-cold NaMops. Incubation of the vesicles with 1 to 10 units of DPCC-treated trypsin approximately doubled Na^+-Ca^{2+} exchange activity (Fig. 5), whereas incubation with a lower (0.01 units) or higher (50 units) amount of DPCC-treated trypsin only slightly increased exchange activity. TLCK-treated α-chymotrypsin (1 unit) increased exchange activity similarly to trypsin (Table I-A). In contrast to the effect of trypsin on vesicles prepared with the proteinase inhibitors, trypsin (0.1 and 1 unit) had no effect on exchange activity of F1 vesicles prepared in the absence of the proteinase inhibitors (two experiments, data not shown). This observation suggests that endogenous proteolysis activates exchange by a similar mechanism as trypsin.

The sarcolemmal vesicles apparently have an endogenous proteinase which increases exchange activity because removing the inhibitors was sufficient to double exchange activity (Table I-A, B). The proteinase inhibitors were removed by diluting the F1 fraction with NaMops prior to centrifugation. After centrifugation the vesicles were suspended with NaMops and incubated at 37°C for 20 min (Table I-B). A somewhat larger increase in exchange activity was obtained by diluting the vesicles without proteinase inhibitors and incubating them with 5 units of DPCC-treated trypsin (Table I-B).

Depleting cellular ATP has no effect on Na^+-Ca^{2+} exchange activity

Because depleting cellular ATP with metabolic poisons strongly decreases exchange activity in intact aortic myocytes [19], we examined exchange activity of SN2P vesicles prepared by Dounce homogenization in the presence of the proteinase inhibitors from ATP-depleted and control cells. The cultures were rinsed and incubated with a physiological salts solution which contained (in mM): 120 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$ and 20 Hepes/Tris (pH 7.4). The cultures were incubated for 1.5 h in this solution which contained 10 mM glucose or 2 mM 2-deoxy-D-glucose plus 2 μM rotenone to deplete cellular ATP [19]. The vesicles from the ATP-depleted cells had the same exchange activity as those from cells with normal ATP (0.31 ± 0.06 compared to 0.30 ± 0.08 nmol $^{45}Ca^{2+}$/30 s per mg protein, $n = 5$ experiments). Adding 5 mM MgATP to the vesicles from control or ATP-depleted cells also had no effect on exchange activity (Lyu, unpublished data). Additionally, the vesicles were incubated in the presence and absence of 10 μM $CaCl_2$ and concentrated cytosol as well as inhibitors of protein phosphatases (NaF, Na_3VO_4 and okadaic acid) in the presence and absence of 5 mM MgATP. None of the treatments had any affect on exchange activity. Because neither ATP-depletion, nor in vitro treatment with MgATP affected Na^+-Ca^{2+} exchange activity, it appears that the exchanger in vesicles is deregulated with respect to dependence on metabolic energy.

Discussion

Here we have shown that including five proteinase inhibitors in all of the solutions used to purify plasma membrane vesicles from aortic myocytes strongly decreased the V_{max} of Na^+-Ca^{2+} exchange without affecting its K_m for Ca^{2+}. Adding the proteinase inhibitors to vesicles prepared without them, however, had no effect on the exchange activity (Fig. 2) indicating that the inhibitors do not directly affect exchange activity. The proteinase inhibitors also had no effect on membrane purity as indicated by 5'-nucleotidase activity. Removing the proteinase inhibitors and adding trypsin or chymotrypsin strikingly increased exchange activity providing the vesicles were prepared with the proteinase inhibitors (Fig. 5). Trypsin or endogenous proteolysis apparently activates exchange by a similar mechanism because trypsin had no effect on exchange activity of F1 vesicles prepared in the absence of the proteinase inhibitors.

Intact cells have a much higher V_{max} (~ 10 nmol/15 s per mg, Fig. 3 in Ref. 19) than the plasma membrane vesicles prepared in the presence of proteinase inhibitors (1 nmol/15 s per mg). Furthermore, the vesicles have approx. 7-times higher 5'-nucleotidase activity and approx. 6-times higher specific exchange activity than the crude homogenate. Therefore, the V_{max} of exchange activity in plasma membrane vesicles is about a sixtieth of the expected value. Activation of the exchanger by limited proteolytic cleavage during membrane purification apparently restores some of the activity which is lost during membrane purification. Because no method is available for counting Na^+-Ca^{2+} exchangers, it is not possible to distinguish an unmasking of latent exchangers from the stimulation of exchange activity.

It is noteworthy that the initial rate of exchange activity in the vesicles was probably somewhat underestimated, especially at higher Ca^{2+} concentrations, because the Na^+ gradient dissipates rapidly (Fig. 4) and a 15 s interval was used to assay exchange activity. The time-course of $^{45}Ca^{2+}$ uptake at 100 μM Ca^{2+} by F1 vesicles prepared without the proteinase inhibitors indicated that a 15 s uptake underestimated the initial exchange rate by about 20% compared to a 5 s uptake (Lyu, unpublished data). This moderate underestimation of the initial exchange rate would lead to an overestimation of the K_m and an underestimation of the V_{max}, but would not invalidate our major conclusions concerning the large loss of exchange activity during vesicle preparation and activation by endogenous proteolysis.

Recently, Hilgemann [16] observed that adding chymotrypsin to the cytoplasmic surface of giant excised, inside-out patches of sarcolemma from guinea-pig heart cells increased the Na^+-Ca^{2+} exchange current. Furthermore, the chymotrypsin treatment abolished the modulatory influences of Ca^{2+} and MgATP on the exchange current [16]. Proteolysis may also deregulate the exchanger in vesicles from aortic myocytes because vesicles from control and ATP-depleted cells have the same exchange activity even though ATP-depletion strongly inhibits the exchanger in intact aortic myocytes [19].

Previously, Philipson and Nishimoto [34] and Wakabayashi and Goshima [35] showed that exogenous proteases increase exchange activity in cardiac membrane vesicles. Proteinase treatment decreased the K_m for Ca^{2+} from 22 to 8 μM and had no effect on the V_{max} of the exchanger in cardiac membrane vesicles [34]. This contrasts with the present findings that endogenous proteolysis increased the V_{max} without affecting the K_m in vesicles from aortic myocytes. The finding by Slaughter and coworkers [12] that chymotrypsin had no effect on exchange activity in sarcolemmal vesicles from bovine tracheal smooth muscle also contrasts with the present data. It is unclear to what extent endogenous proteolysis influences exchange activity in sarcolemmal vesicles from cardiac or smooth muscle tissues and limits the extent of the activation by exogenous proteolysis.

The recent cloning of the Na^+-Ca^{2+} exchanger [36] makes it possible to elucidate structural differences in the exchanger in cardiac and smooth muscle and sites of proteinase cleavage. Whether the loss of exchange activity during the preparation of vesicles from aortic myocytes is caused by the loss of a regulatory subunit or a chemical change in the exchanger itself or both remains to be investigated. Interestingly, the cardiac Na^+-Ca^{2+} exchanger has a region with interspersed hydrophobic and basic residues, which is characteristic of calmodulin binding domains, and a peptide with this sequence potently inhibits Na^+-Ca^{2+} exchange activity in sarcolemmal vesicles and excised patches [36,37]. Conceivably, the calmodulin binding domain of the cardiac exchanger may have an autoinhibitory role [37]. The present findings raise the possibility that a limited proteolysis of the exchanger during membrane isolation from muscle tissues partially masks the loss of activity caused by deregulation of the exchanger. Although an accurate assessment of Na^+-Ca^{2+} exchange activity in intact tissues is not currently possible, the exchanger in sarcolemmal vesicles may now be examined for proteolytic nicks that might activate and/or deregulate it to further assess the role of the putative autoinhibitory domain.

Acknowledgements

We thank Lucinda Smith and Yingxin Zhuang for providing the cell cultures. This work was supported by grants DK39258 and Research Career Development Award HL01671 from the National Institutes of Health and a grant-in-aid from the American Heart Association with funds contributed by the Central Florida Region.

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