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Regulatory Processes on the Cytoplasmic Surface of the Na^+/Ca^{2+} Exchanger from Lobster Exoskeletal Muscle

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Abstract. A partially purified preparation of the lobster muscle Na^+/Ca^{2+} exchanger was reconstituted with, presumably, random orientation in liposomes. Ca^{2+} efflux from ^{45}Ca-loaded vesicles was studied in exchanger molecules in which the transporter cytoplasmic surface was exposed to the extravesicular (*ev*) medium. Extravesicular Na^+ (Na_{ev})-dependent Ca^{2+} efflux depended directly upon the extravesicular Ca^{2+} concentration ($[Ca^{2+}]_{ev}$) with a half-maximal activation at $[Ca^{2+}]_{ev} = 0.6$ μM. This suggests that the lobster muscle exchanger is catalytically upregulated by cytoplasmic Ca^{2+}, as in most other species. In contrast, at low $[Na^+]_{ev}$, the Ca_{ev}-binding site (i.e., on the cytoplasmic surface) for Ca^{2+} transported via Ca^{2+}/Ca^{2+} exchange was half-maximally activated by about 7.5 μM Ca^{2+}. Mild proteolysis of the Na^+/Ca^{2+} exchanger by α-chymotrypsin also upregulated the Na_{ev}-dependent Ca^{2+} efflux. Following proteolytic digestion in Ca-free medium, the exchanger was no longer regulated by nontransported *ev* Ca^{2+}. Proteolytic digestion in the presence of 1.9 μM free *ev* Ca^{2+}, however, induced only a 1.6-fold augmentation of Ca^{2+} efflux, whereas, after digestion in nominally Ca-free medium, a 2.3-fold augmentation was observed; Ca^{2+} also inhibited proteolytic degradation of the Na^+/Ca^{2+} exchanger measured by immunoblotting. These data suggest that Ca^{2+}, bound to a high affinity binding site, protects against the activation of the Na^+/Ca^{2+} exchanger by α-chymotrypsin. Additionally, we observed a 6-fold increase in the Na^+/Ca^{2+} exchange rate, on average, when the intra- and extravesicular salt concentrations were increased from 160 to 450 mM, suggesting that the lobster muscle exchanger is optimized for transport at the high salt concentration present in lobster body fluids.

Key words: Catalytic regulation — Ionic strength — α-chymotrypsin; — Protection from proteolytic digestion

Introduction

Plasmalemmal Na^+/Ca^{2+} exchange is one of the main mechanisms by which most cells in higher animals regulate their cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$). The exchanger utilizes energy from the transplasmalemmal Na^+ electrochemical gradient ($\Delta\bar{\mu}_{Na}$) to extrude net Ca^{2+} from the cells, by exchanging (in most cell types) one Ca^{2+} ion for three Na^+ ions [3]. The Na^+/Ca^{2+} exchanger also can operate in reverse (i.e., transport net Ca^{2+} into cells) when $\Delta\bar{\mu}_{Na}$ is reduced by depolarization, and/or by a rise in the intracellular Na^+ concentration, as may occur with inhibition of the Na^+ pump [3].

During an investigation of the net Ca^{2+} influx or "reversed" mode of exchanger operation in dialyzed squid axons, DiPolo [5] observed that low concentrations of cytoplasmic Ca^{2+} paradoxically augmented the cytoplasmic Na^+ (Na_i)-dependent uptake of Ca^{2+} into the axons. Subsequently, several studies confirmed the existence of this regulatory ("catalytic") Ca^{2+} binding site on Na^+/Ca^{2+} exchangers in vertebrates and invertebrates and demonstrated that it is different from the intracellular Ca^{2+} transport site [3, 6, 8, 18, 20, 26, 30, 31]. In most cases the affinity for Ca^{2+} at the regulatory binding site was higher (apparent dissociation constant, $K_d < 1$ μM) than at the cytoplasmic transport site ($K_d > 1$ μM) [6, 7, 26].[1]

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[1]We employ the term catalytic, to describe the action of a ligand that modulates (i.e., up- or downregulates) the transport process but that is not, itself, "consumed" or transported. Thus, we distinguish between

The Na^+/Ca^{2+} exchanger also can be activated by low concentrations of α-chymotrypsin [12, 14, 29]. Mild proteolysis cleaves the large cytoplasmic loop of the Na^+/Ca^{2+} exchanger that contains several regulatory sites, including the catalytic Ca^{2+} binding site [22, 26]. In the absence of cytoplasmic Ca^{2+}, this site downregulates the exchanger; binding of Ca^{2+} then activates the exchanger [6, 18, 30, 31].

The intracellular regulatory Ca^{2+}-binding site on the Na^+/Ca^{2+} exchanger from *Drosophila melanogaster* expresssed in *Xenopus* oocytes apparently behaves differently [19]: cytoplasmic Ca^{2+} inhibits the Na_i-dependent influx of Ca^{2+} from the extracellular fluid. This inhibitory effect was almost complete at $[Ca^{2+}]_i \cong 3$ μM. The amino acid sequences of the proposed regulatory Ca^{2+} binding domains in *Drosophila*, an arthropod, and in mammals are, however, almost identical. Hryshko et al. [19] therefore hypothesized that the intramolecular signal transduction mechanism is altered in *Drosophila*, perhaps because of alternative splicing.

Here we describe an investigation of the cytoplasmic Ca^{2+} regulatory site in the Na^+/Ca^{2+} exchanger system of another arthropod, namely, in the exoskeletal muscle of the lobster. This is noteworthy because barnacle (also an arthropod) exoskeletal muscle exhibits a more orthodox, stimulatory catalytic Ca^{2+}-binding site [30, 31].

Previously, we used a partially purified and reconstituted vesicle preparation of Na^+/Ca^{2+} exchanger from lobster muscle to investigate the rheogenic properties of this type of transporter with the planar lipid bilayer technique [11]. In the present work we loaded the reconstituted proteoliposomes with ^{45}Ca to study the influence of Ca^{2+}, presented at the cytoplasmic (extravesicular, *ev*) surface of the exchanger, on Na_{ev}-dependent[2] and Na_{ev}-independent Ca^{2+} efflux from the vesicles.

Materials and Methods

Proteoliposome Preparation

Lobsters (*Homarus americanus*) were purchased from a local seafood wholesaler. Preparation of sarcolemmal membranes and subsequent partial purification and reconstitution of the Na^+/Ca^{2+} exchanger protein into liposomes were performed to a modification of a published protocol [33, 10], as described [11]. The exchanger in these reconstituted proteoliposomes was approximately 50% pure.

^{45}Ca Efflux Measurements

Na_{ev}-dependent and Na_{ev}-independent components of the Ca^{2+} efflux from the proteoliposomes were measured as described [28] with minor modification (Fig. 1). Proteoliposomes were filled with 160 mM NaCl, 20 mM TRIS-HCl (pH 7.5). Aliquots (5 μl) were diluted into 75 μl of 160 mM KCl, 20 mM TRIS-HCl (pH 7.5) containing 25 μM $CaCl_2$ (labeled with 0.15 μCi ^{45}Ca) and incubated at room temperature (22–24°C) for 1 min to load the vesicles with ^{45}Ca. The efflux of ^{45}Ca was induced by diluting the entire 80 μl sample with 960 μl of NaCl buffer which contained 160 mM NaCl and 20 mM TRIS-HCl (pH 7.5). The desired free Ca^{2+} concentrations in these solutions ($[Ca^{2+}]_{ev}$) were obtained by adding EGTA (ethylene glycol-O,O′-bis (2-aminoethyl)-N,N,N′,N′-tetraacetic acid) and $CaCl_2$ in the appropriate ratios (*see below*). For controls, ^{45}Ca-loaded proteoliposomes were diluted with 960 μl of KCl buffer consisting of 160 mM KCl, 20 mM TRIS-HCl (pH 7.5) with the appropriate free Ca^{2+} concentrations. Following incubation for 2 or 10 sec at 22–24°C, 200 μl samples were loaded onto 1.25 ml columns of CM-Sepharose (Pharmacia Biotech, Freiburg, FRG) that had been equilibrated with either the NaCl or KCl buffer, as appropriate, to remove *ev* Ca^{2+} ions. The columns were rapidly washed four times with 200 μl of the appropriate (KCl or NaCl) buffer. The vesicles passed through the columns by gravity flow in ≈15 sec. The eluate was mixed with 6 ml of scintillation cocktail (Quicksafe A; Zinsser Analytic, Frankfurt, FRG) and counted (Canberra-Packard, model 1500 Liquid Scintillation Analyzer, Dreieich, FRG). The counts correspond to the ^{45}Ca retained in the vesicles. The difference between the counts from the samples diluted into KCl buffer, and into NaCl buffer, corresponds to the Na_{ev}-dependent Ca^{2+} efflux from the proteoliposomes. This efflux protocol is thus the reverse of common ^{45}Ca uptake experiments [32].

It made no difference whether NaCl- or KCl-equilibrated columns and wash buffers were used (Table 1). Loading the vesicles with ^{45}Ca to equilibrium, at the expense of the Na^+ gradient, apparently needed only a small fraction of the Na_{iv}, so that after the brief Ca^{2+} efflux experiment (which is actually a Na^+ reloading process) $[Na]_{iv}$ was still close to 160 mM. Thus, the vesicles were not very leaky to either Na^+ or K^+.

The free Ca^{2+} concentrations of the buffers were calculated with a custom computer program using published stability constants and binding enthalpy values [25]. Contaminating Ca^{2+} in the buffers, determined by atomic absorption spectroscopy (model AAS-5000, Perkin-Elmer, Überlingen, FRG), was included when calculating the free Ca^{2+} concentrations.

^{45}Ca Uptake (Load) Measurements

Na^+ gradient-dependent ^{45}Ca uptake was performed as described [32]. Na^+-filled proteoliposomes (5 μl) were incubated for 1 sec to 1 min at 22–24°C in KCl buffer containing 160 mM KCl, 20 mM TRIS-HCl (pH 7.5) and the desired concentration of free Ca^{2+}. The vesicles were then filtered through CM-sepharose columns as described above. In these experiments, control vesicles were incubated in 160 mM NaCl buffer (i.e., in the absence of an outwardly directed Na^+ gradient). The difference between the ^{45}Ca uptake from the KCl buffer and from the NaCl buffer is the Na^+ gradient dependent Ca^{2+} uptake.

^{45}Ca Efflux Measurements at High Salt (Concentrations)

As noted in Results, some Ca^{2+} efflux experiments were performed under high salt conditions. In these experiments, all of the intra- and

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the catalytic and the transport sites to which Ca^{2+} binds at the cytoplasmic surface of the exchanger. To simplify the discussion concerning three distinct Na^+/Ca^{2+} exchanger Ca^{2+} binding sites, we introduce the following nomenclature for the apparent Ca^{2+} dissociation constants: $K_{d(Ca)ext} = K_d$ at the transport site on the extracellular surface; $K_{d(Ca)cyt} = K_d$ at the transport site on the cytoplasmic surface; $K_{d(Ca)reg} = K_d$ at the catalytic or regulatory (nontransport) site on the cytoplasmic surface.

[2]Subscripts *ev* and *iv* refer to extravesicular and intravesicular media, respectively.

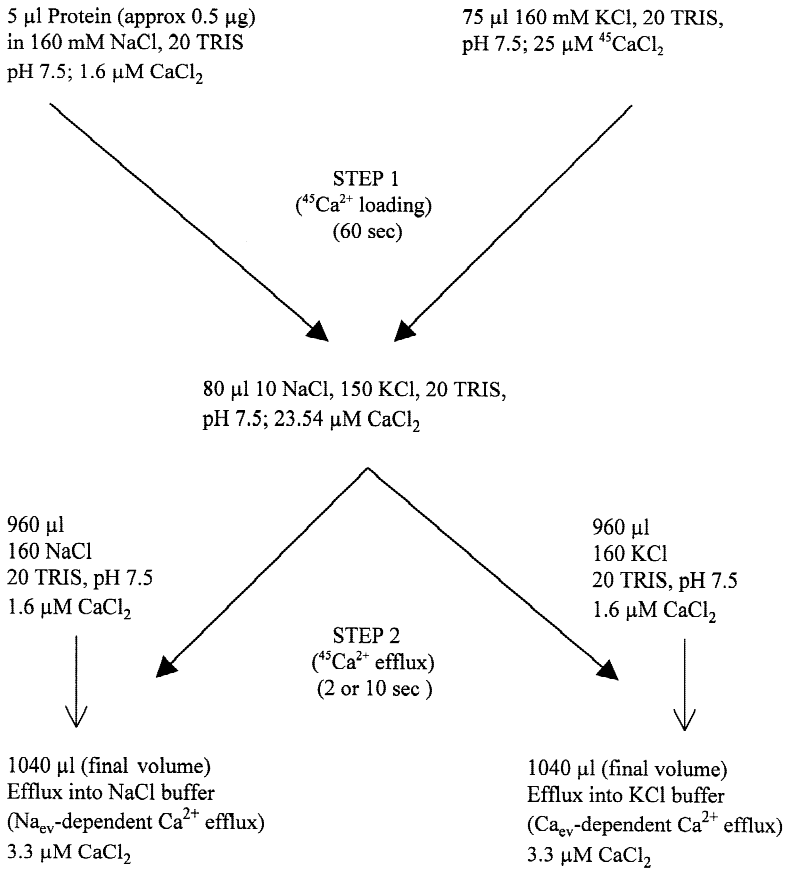

Fig. 1. Schematic representation of the sequence of steps used for the $^{45}Ca^{2+}$ efflux experiments. The Ca^{2+} concentrations were taken from a representative experiment. Ca^{2+} concentrations were determined for each newly prepared solution by atomic absorption spectroscopy.

Table 1. Effect of the ionic composition (NaCl or KCl) of the solutions used for column pre-equilibration and for washing on the residual ^{45}Ca load in proteoliposomes containing the Na^+/Ca^{2+} exchanger.

		Relative ^{45}Ca load (% of control)
Control	K-vesicles on K-column	100
Test 1	Na-vesicles on Na-column	73.6 ± 4.4 (n = 13)
Test 2	Na-vesicles on K-column	72.6 ± 6.4 (n = 14)

Control = residual ^{45}Ca content of vesicles incubated in KCl solution and washed on a KCl-equilibrated column (set to 100%). Two types of test vesicles are shown: residual ^{45}Ca content of vesicles incubated for 2 sec in NaCl solution and washed with NaCl solution on a NaCl-equilibrated column (Test 1) and residual ^{45}Ca content of vesicles incubated for 2 sec in NaCl solution and washed with KCl solution on a KCl-equilibrated column (Test 2). The incubation solutions contained 1.5 µM Ca^{2+}.

extravesicular 160 mM NaCl or 160 M KCl solutions were replaced by 450 mM solutions which are isotonic for marine invertebrates [30, 31].

Proteolysis Experiments

In some experiments, the proteoliposomes were subjected to limited proteolysis. An aliquot (0.5 µl) of a protease stock solution containing 15 µg/µl of either α-chymotrypsin or trypsin was added to 30 µl of the vesicle suspension to obtain a final protease concentration of 0.25 µg/µl. The mixed samples were incubated for 60 or 120 min and then tested for Ca^{2+} efflux as described above; e.g., 1 min loading with ^{45}Ca followed by a 10-sec efflux. Incubation in the absence of Ca_{ev} was assured by including 10 µM EGTA (0.5 µl of a 600 µM EGTA stock per 30 µl vesicles); the appropriate amount of NaCl buffer was added to the control samples.

Immunoblotting

Aliquots of proteoliposome suspensions (150 ml containing 7.5 µg of protein) were subjected to limited proteolysis in the presence or absence of 10 µM EGTA as described in the preceding section. α-Chymotrypsin activity was stopped after 120 min incubation (22–24°C) by addition of 2 ml of 12.5% trichloroacetic acid. the precipitated samples were centrifuged at 5,000 × g for 30 min. The lipids were extracted from pelleted proteoliposomes and the proteins were separated by SDS-PAGE [11]. Separated proteins were transferred to a Hybond-C Extra nitrocellulose membrane (Amersham Life Sciences, Arlington Heights, IL). Membranes were blocked with 5% nonfat dry milk in TBS-T (tris(hydroxymethyl)-aminomethane; (TRIS)-buffered saline (137 mM NaCl, 20 mM TRIS, pH 7.6 with 0.1% Tween-20) for 1 hr at 22–24°C. The blots were then incubated overnight at room temperature with polyclonal antibodies (1:500 dilution in TBS-T) raised against purified dog cardiac Na^+/Ca^{2+} exchanger (from Dr. G.E. Lindenmayer, Med. Univ. S. Carolina). The membranes were washed three times for 10 min each in TBS-T and incubated with anti-rabbit

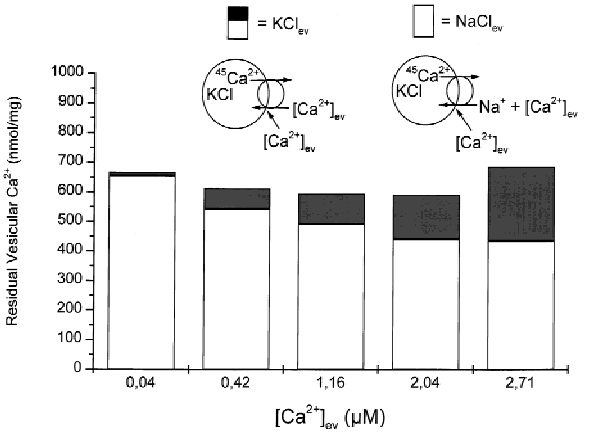

Fig. 2. Data from a representative $^{45}Ca^{2+}$ efflux experiment (*see* Materials and Methods). Each bar represents the results of paired test ($NaCl_{ev}$) and control (KCl_{ev}) measurements. The total bar height corresponds to the total residual Ca^{2+} content of proteoliposomes that were preloaded with $^{45}Ca^{2+}$, and then incubated for 2 sec with a 160 mM KCl solution containing the free Ca^{2+} concentration indicated below each bar (*see* diagram at upper left). $[Ca^{2+}]_{ev}$ was reduced to ≈50 nM by adding 10 μM EGTA to the solutions containing 3.3 μM Ca^{2+} (*see* Fig. 1). The white portion of the bar corresponds to the Ca^{2+} remaining after the 2 sec incubation with 160 mM NaCl solution containing the same free Ca^{2+} concentration (*see* diagram at upper right). The filled area at the top of each bar thus represents the Na_{ev}-dependent loss of $^{45}Ca^{2+}$ from the proteoliposomes. This and all subsequent experiments were performed at room temperature (22–24°C). In the diagrams in this and the subsequent figures, the large circles correspond to the vesicles and the small circles correspond to the exchanger. Horizontal arrows show the direction of transport of the indicated ion species; upward diagonal arrows indicate the agents involved in activation of the exchanger.

horseradish peroxidase-conjugated IgG for 1 hr. An enhanced chemiluminescence detection system (ECL; Amersham Life Sciences, Arlington Heights, IL) was used to detect bound antibodies. The films were scanned using a ScanMaker III scanner (Mikrotec Lab, Retondo, CA) and NCX band intensities were analyzed by MetaMorph imaging software (Universal Imaging Corporation, West Chester, PA).

Reagents

Electrolyte salts and buffers were obtained from Sigma (Deisenhofen, FRG, and St. Louis, MO), Fluka (Buchs, Switzerland) or Merck (Darmstadt, FRG), and were analytical grade. ^{45}Ca was purchased from Amersham Buchler (Braunschweig, FRG). Trypsin (Type III) and α-chymotrypsin (Type I-S), both from bovine pancreas, and soybean trypsin/α-chymotrypsin (Bowman-Birk) inhibitor were obtained from Sigma, as was the soybean lecithin used for reconstitution of the proteoliposomes.

Statistics

Data are shown as mean values ± SEM for *n* determinations. *P* values were determined by Student's *t* test for unpaired or paired data, where appropriate. Each type of experiment was performed a minimum of three times, on a total of at least two different proteoliposome preparations. Curves were fitted using Microcal Origin version 4.1 (Microcal Software, Northampton, MA).

Results

^{45}Ca Uptake into Proteoliposomes

To investigate the existence and properties of a regulatory Ca^{2+}-binding site on the lobster muscle Na^+/Ca^{2+} exchanger, proteoliposomes reconstituted with partially purified exchanger were loaded with ^{45}Ca (Materials and Methods). The total Ca^{2+} content of the vesicles following the 1 min loading period in 160 mM KCl buffer containing 25 μM free Ca^{2+} was, typically, between 600 and 900 nmol Ca/mg protein (as ^{45}Ca). For comparison, the Na^+ gradient-dependent ^{45}Ca uptake from the low Na^+ medium was about 60–80 nmol/mg protein after 1 sec incubation (*not shown*). The rate of ^{45}Ca uptake slowed considerably after the initial few seconds of incubation; after 1 min, the specific activity of the *iv* Ca^{2+} was presumed equal to that of the *ev* Ca^{2+}.

$^{45}Ca^{2+}$ Efflux from Proteoliposomes

Ca^{2+} efflux from the ^{45}Ca-loaded proteoliposomes was initiated by incubating the vesicles in KCl buffers with various free Ca^{2+} concentrations to induce Ca_{ev}-dependent ^{45}Ca efflux (i.e., the Ca^{2+}/Ca^{2+} exchange mode of the Na^+/Ca^{2+} exchanger [3]). Alternatively, Ca^{2+} efflux was activated by incubating the vesicles in NaCl buffers with various free Ca^{2+} concentrations to induce Na_{ev}-dependent ^{45}Ca efflux together with the inevitable Ca_{ev}-dependent ^{45}Ca efflux. The difference between the fluxes in the KCl and NaCl buffers at a given free Ca^{2+} concentration corresponds to the Na_{ev}-dependent component of the total Ca^{2+} efflux (i.e., Na^+/Ca^{2+} exchange).

Figure 2 shows data from a representative ^{45}Ca efflux experiment. The ordinate indicates the residual Ca^{2+} content of the vesicles following a 2 sec efflux period. The total height of each bar corresponds to the amount of vesicular ^{45}Ca load remaining after the incubation in 160 mM KCl buffer with the Ca_{ev} concentration indicated on the abscissa. The height of each open bar shows the amount of the ^{45}Ca load remaining after the incubation in 160 mM NaCl buffer. The filled area at the top of each bar is the Na_{ev}-dependent (Na^+/Ca^{2+} exchange) component of the ^{45}Ca efflux.

Figure 3 shows the mean Na_{ev}-dependent component of the ^{45}Ca efflux from six experiments (*see* Fig. 2) graphed as a function of $[Ca^{2+}]_{ev}$. The Na_{ev}-dependent ^{45}Ca efflux is activated by increasing $[Ca^{2+}]_{ev}$, which corresponds to catalytic activation at a site on the cytoplasmic surface of the Na^+/Ca^{2+} exchanger. Increasing $[Ca^{2+}]_{ev}$ from 40 nM to 2.71 μM (i.e., near saturation) stimulated Na_{ev}-dependent Ca^{2+} efflux by a factor of about 10, with apparent half-maximal activation at

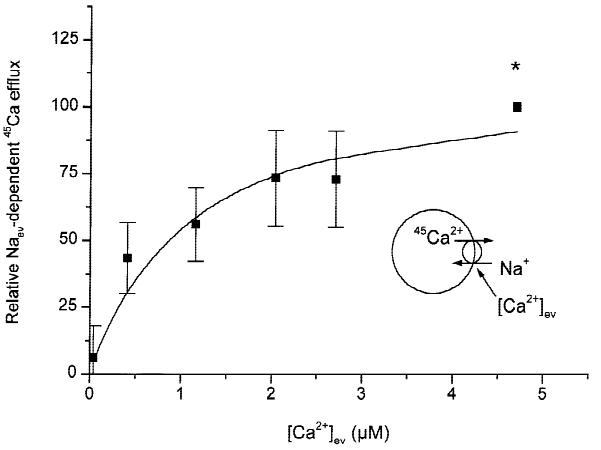

Fig. 3. Relative Na_{ev}-dependent Ca^{2+} efflux (J) from ^{45}Ca-loaded proteoliposomes, graphed as a function of $[Ca^{2+}]_{ev}$; the diagram at the lower right indicates the measurement conditions. Each data point represents the mean value from four experiments ± SE. The data were fitted to the Michaelis-Menten equation:

$$J = (J_{max} \cdot [Ca^{2+}]_{ev})/K_{d(Ca)reg} \cdot [Ca^{2+}]_{ev})$$

J is the observed Na_{ev}-dependent Ca^{2+} efflux and J_{max} is the maximal efflux. The calculated $K_{d(Ca)reg}$ was 0.86 μM; J_{max} was 107% of the flux at 4.7 μM Ca^{2+}, and corresponds to a Ca^{2+} efflux of 133 ± 43 nmol/sec. The data were obtained from six experiments like that of Fig. 2. The asterisk indicates the data point to which all other data were normalized.

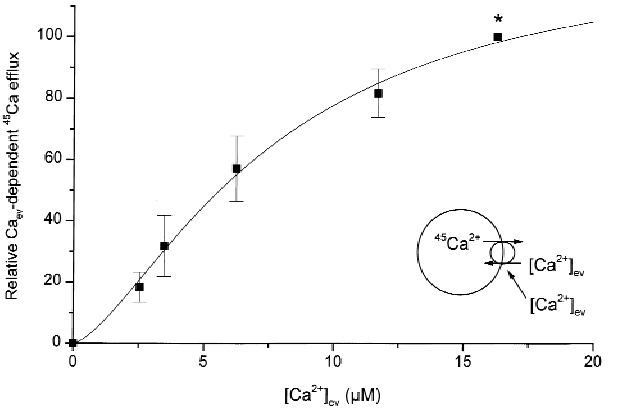

Fig. 4. Relative Ca_{ev}-dependent Ca^{2+} efflux (Ca^{2+}/Ca^{2+} exchange) from proteoliposomes. This efflux corresponds to the difference between the total Ca^{2+} content of the proteoliposomes (i.e., total bar height from experiments similar to the one in Fig. 2) at $[Ca^{2+}]_{ev}$ = 0.05 μM and at higher $[Ca^{2+}]_{ev}$ (*shown* on the abscissa) following a 10-sec incubation. The diagram at the lower right shows the measurement conditions and indicates the ambiguity of the determination of $K_{d(Ca)}$ because Ca^{2+} binds at two sites. At $[Ca^{2+}]_{ev}$ = 0.05 μM, the Ca_{ev}-dependent Ca^{2+} efflux was assumed to be negligible. The data are the means ± SE from five experiments. The data were fitted to a Hill equation with the parameters, $K_{(Ca)cyt}$ = 7.6 ± 2.3 μM and Hill coefficient, n_H = 1.5 ± 0.3. The asterisk indicates the data point to which all other data were normalized. The maximal Ca^{2+} efflux was 2.2 ± 0.3 nmol/sec.

$[Ca^{2+}]_{ev}$ = 0.86 μM (i.e., $K_{d(Ca)reg}$ = 0.86 μM). Similar results ($K_{d(Ca)reg}$ = 0.6 μM) were obtained when the efflux period was extended from 2 sec to 10 sec (*not shown*).

The Na_{ev}-independent, Ca_{ev}-dependent Ca^{2+} efflux could also, in principle, be determined from the data in Fig. 2, but this efflux was much smaller than the Na_{ev}-dependent Ca^{2+} efflux. Therefore, we used a 10 sec rather than a 2 sec efflux. Also, we assumed that the Ca^{2+} efflux into media with 50 nM Ca^{2+} corresponded to the efflux at nominal 0 mM Ca_{ev}, which is reasonable if $K_d > 1$ μM (*see below*). Accordingly, the Ca_{ev}-dependent Ca^{2+} efflux corresponds to the difference between the total vesicular Ca^{2+} content at $[Ca^{2+}]_{ev}$ = 50 nM (the "baseline"), and the residual Ca^{2+} content at the higher $[Ca^{2+}]_{ev}$ (*see* Fig. 2). Higher EGTA concentrations (>>10 μM) could not be used because the Ca-EGTA complexes then passed through the CM-Sepharose column and raised the background counts (*not shown*).

Figure 4 shows the Ca_{ev}-dependent Ca^{2+} efflux into KCl buffers (i.e., Ca^{2+}/Ca^{2+} exchange) graphed as a function of $[Ca^{2+}]_{ev}$. The apparent Ca^{2+} dissociation constant ($K_{d(Ca)cyt}$) is 7.6 μM. The maximum rate of Ca^{2+} transport, 2.2 nmol/mg protein per sec, is substantially smaller than the maximal Na_{ev}-dependent Ca^{2+} efflux, but may be underestimated slightly because $[Ca^{2+}]_{ev}$ could not be reduced below 50 nM (*see above*). The curve has a Hill coefficient of 1.5, which suggests some cooperativity between Ca^{2+} ions.

Effects of Proteolytic Enzymes on Ca^{2+} Efflux

Results from proteolysis experiments are presented in Figs. 5–8. Figure 5*A* shows that the Na_{ev}-dependent Ca^{2+} efflux was markedly augmented both by $[Ca^{2+}]_{ev}$ = 4.0 μM before proteolysis (second bar), and by a 120 min incubation with 0.25 μg/μl α-chymotrypsin at room temperature and very low $[Ca^{2+}]_{ev}$ (30 nM, third bar). Addition of Ca_{ev} (4 μM) after the incubation with α-chymotrypsin had no additional effect on the Na_{ev}-dependent Ca^{2+} efflux. Thus, either the addition of Ca_{ev}, or proteolysis of the large cytoplasmic loop of the exchanger (where the catalytic Ca^{2+}-binding site is located [22, 26]), disinhibited the exchanger [3] and thereby augmented the efflux. The ^{45}Ca load of the vesicles prior to efflux was not markedly different in untreated vesicles and those treated with α-chymotrypsin; thus, the 1 min loading time was sufficient to achieve a maximum ^{45}Ca load even with an unstimulated exchanger.

The data in Fig. 5 reveal some other remarkable features of the lobster muscle exchanger. As illustrated here, the Na_{ev}-dependent Ca^{2+} efflux was increased about 2.5-fold (average = 2.30 ± 0.24, n = 5) by the proteolysis, and to a comparable extent by Ca_{ev} (average

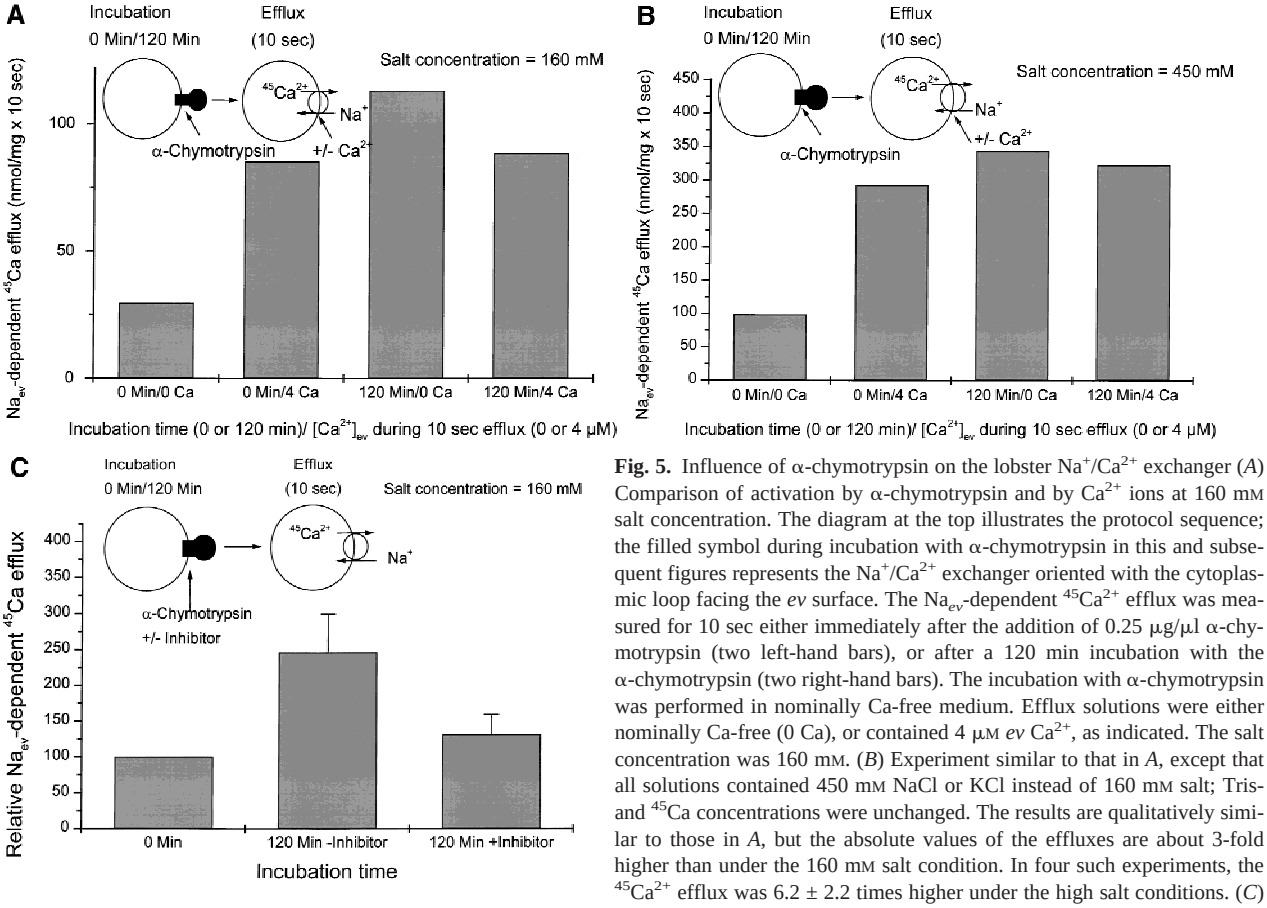

Fig. 5. Influence of α-chymotrypsin on the lobster Na^+/Ca^{2+} exchanger (*A*) Comparison of activation by α-chymotrypsin and by Ca^{2+} ions at 160 mM salt concentration. The diagram at the top illustrates the protocol sequence; the filled symbol during incubation with α-chymotrypsin in this and subsequent figures represents the Na^+/Ca^{2+} exchanger oriented with the cytoplasmic loop facing the *ev* surface. The Na_{ev}-dependent $^{45}Ca^{2+}$ efflux was measured for 10 sec either immediately after the addition of 0.25 μg/μl α-chymotrypsin (two left-hand bars), or after a 120 min incubation with the α-chymotrypsin (two right-hand bars). The incubation with α-chymotrypsin was performed in nominally Ca-free medium. Efflux solutions were either nominally Ca-free (0 Ca), or contained 4 μM *ev* Ca^{2+}, as indicated. The salt concentration was 160 mM. (*B*) Experiment similar to that in *A*, except that all solutions contained 450 mM NaCl or KCl instead of 160 mM salt; Tris- and ^{45}Ca concentrations were unchanged. The results are qualitatively similar to those in *A*, but the absolute values of the effluxes are about 3-fold higher than under the 160 mM salt condition. In four such experiments, the $^{45}Ca^{2+}$ efflux was 6.2 ± 2.2 times higher under the high salt conditions. (*C*) Addition of trypsin-chymotrypsin (Bowman-Birk) inhibitor prevents the activation of $^{45}Ca^{2+}$ efflux by α-chymotrypsin. The diagram at the top shows the protocol (all incubation solutions were nominally Ca-free). The difference between the middle bar (120 min incubation with α-chymotrypsin minus inhibitor) and the left-hand (0 min incubation) bar corresponds to the activation of Na_{ev}-dependent ^{45}Ca efflux (measured for 10 sec) in media containing 10 μM EGTA and 0.25 μg/μl α-chymotrypsin, as in *A*. The addition of 0.5 μg/μl of the inhibitor during the 120 min incubation (right-hand bar) suppressed the stimulatory effect of the α-chymotrypsin. All experiments were performed at room temperature (22–24°C).

= 2.81 ± 1.04, $n = 4$). Qualitatively similar results were obtained with buffers containing 160 mM salt concentrations (≈isotonic for mammalian blood plasmas; Fig. 5*A*) and with buffers containing 450 mM salt concentrations (≈isotonic for marine invertebrate hemolymph; Fig. 5*B*). The absolute magnitudes of the Ca^{2+} fluxes were, however, markedly different. At the lower salt concentration (Fig. 5*A*), the maximal Ca^{2+} efflux following mild proteolysis was about 120 nmol/mg protein × 10 sec, whereas, at the higher salt concentration (Fig. 5*B*) the comparable efflux was about 340 nmol/mg protein × 10 sec. Indeed, in four such experiments, the Na_{ev}-dependent Ca^{2+} efflux in 450 mM salt was, on average, 6.2 ± 2.2 ($n = 4$) times higher than in 160 mM salt. A comparable result was obtained when testing the Na_i-dependent $^{45}Ca^{2+}$ uptake (incubation time = 1 sec) from solutions containing 25 μM labeled Ca^{2+}, uptake at 450 mM salt exceeded that at 160 mM salt by a factor of 3.4 ± 1.4 ($n = 5$). These findings suggest that the lobster exchanger, which normally operates in a high salt environment, functions more efficiently under these conditions than at lower ionic strength.

Figure 5*C* shows data from an important control. In this case, under four salt conditions, the proteoliposomes were incubated with 0.25 μg/μl α-chymotrypsin for only 30 sec (left-hand bar) or for 120 min (center and right-hand bars). When the incubation medium also contained 0.5 μg/μl soybean trypsin-chymotrypsin (Bowman-Birk) inhibitor (right-hand bar) the α-chymotrypsin induced augmentation of the ^{45}Ca efflux was blocked. Thus, the action of the α-chymotrypsin involved proteolysis, and was not a nonspecific effect.

The time course of the activation of Na_{ev}-dependent Ca^{2+} efflux by α-chymotrypsin is illustrated in Fig. 6. The largest fractional increase in Ca^{2+} efflux was observed during the first 60 min of incubation with the enzyme, and the rate of increase slowed considerably during the second 60 min (triangles). When 1.9 μM Ca^{2+} was added to the enzyme (*ev*) solution, however, activation was greatly reduced (Fig. 6, squares). Since much higher Ca^{2+} concentrations (>100 μM) do not interfere with proteolysis by α-chymotrypsin, and are even normally included in proteolysis media [13], inhibition of the proteolysis by Ca^{2+} cannot be explained by direct

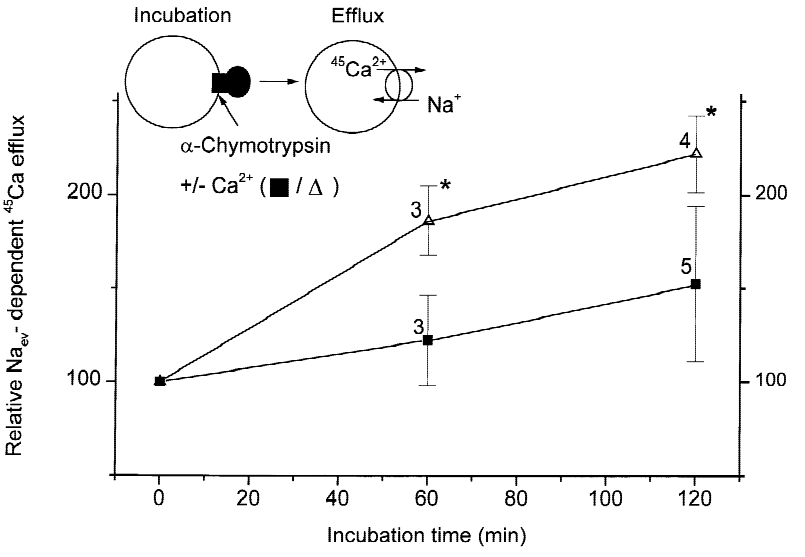

Fig. 6. Time course of activation of the lobster Na^+/Ca^{2+} exchanger by α-chymotrypsin in the presence or absence of *ev* Ca^{2+}. α-Chymotrypsin (0.25 μg/μl) was added at time 0 to two 30 μl aliquots of proteoliposome suspension, one containing 10 μM EGTA (free Ca^{2+} < 0.05 μM; triangles), the other containing 1.9 μM total unbuffered Ca^{2+} (squares). In both samples Na_{ev}-dependent $^{45}Ca^{2+}$ efflux was measured for 10 sec in the presence of 10 μM EGTA, immediately after addition of α-chymotrypsin, and again after 60 and 120 min of incubation at room temperature, respectively (the diagram at the top illustrates the protocol). All data were normalized to 100: efflux activity at time 0. The extent of activation observed with the aliquot digested in medium containing 1.9 μM Ca^{2+} was 122 ± 24% (60 min) and 152 ± 41% (120 min). After incubation in the virtual absence of Ca^{2+}, activation to 186 ± 18% (60 min) and 222 ± 20% (120 min) was detected. Na_{ev}-dependent Ca^{2+} efflux was significantly stimulated by incubating with α-chymotrypsin in Ca-free medium for 60 and 120 min ($P = 0.05$; Student's unpaired *t*-test for the numbers of experiments indicated next to the data points). There was no significant stimulation when the vesicles were incubated with α-chymotrypsin in the presence of 1.9 μM Ca^{2+}.

inhibition of the enzyme. A more likely possibility is that occupation of the catalytic Ca^{2+} binding site by Ca^{2+} may change the conformation of the large cytoplasmic loop of the exchanger so that it is less readily subject to proteolysis (*see below*). This possibility is supported by immunoblot analysis of the α-chymotrypsin treated Na^+/Ca^{2+} exchanger (*see below*).

Other proteases also can stimulate the Na^+/Ca^{2+} exchanger [24, 29]. We tested the effect of 0.5 μg trypsin/μl on the lobster exchanger (Fig. 7). This proteolytic enzyme more than doubled the activity of the exchanger in 60 min, but then began to inhibit the exchanger after a further 60 min incubation. Figure 7 also shows that the activation by trypsin; like that by α-chymotrypsin, also was markedly inhibited by 1.5 μM Ca_{ev}.

At much higher concentrations (10 μg/μl; 30 min incubation), trypsin and α-chymotrypsin reduced the Na^+ gradient-dependent Ca^{2+} uptake (i.e., the Ca^{2+} loading phase) by about 35%. The Na_{ev}-dependent Ca^{2+} efflux from the ^{45}Ca loaded proteoliposomes into media containing 50 nM Ca_{ev} was abolished by this trypsin treatment, and was reduced by about 50% at the high concentration of α-chymotrypsin (*not shown*). Thus, mild proteolysis with low protease concentrations activates the Na^+/Ca^{2+} exchanger, whereas the higher protease concentrations have deleterious effects.

Effects of α-chymotrypsin on the Na^+/Ca^{2+} Exchanger Protein

The protection from proteolytic activation of the exchanger activity by Ca^{2+} raises the question of the possible impact of this phenomenon on the structural integrity of the exchanger protein. This problem was approached by Western blot analysis of the residual exchanger protein before and after limited proteolysis in the presence and absence of Ca^{2+}. Treatment with α-chymotrypsin in 450 mM NaCl for 120 min reduced the intensity of the 120 kDa band that cross-reacted with a polyclonal antibody raised against purified dog heart Na^+/Ca^{2+} exchanger (Fig. 8*A*). When 1.9 μM Ca^{2+} was present during the proteolysis, however, the amount of residual exchanger protein was about twice that observed after digestion in the presence of 10 μM EGTA. Bowman-Birk inhibitor (0.5 mg/ml) completely prevented the proteolysis of the exchanger protein in the absence of Ca^{2+} (*data not shown*). Results from three such experiments, including a Hill coefficient of 1.5 (which suggests some cooperativity between Ca^{2+} ions), are summarized in Fig. 8*B*. After digestion in the presence of 1.9 μM Ca^{2+}, 56 ± 4% of control exchanger protein remained and was detectable, whereas in the absence of protecting Ca^{2+} (i.e., with 10 μM EGTA), only 27 ± 2% was detectable. These results show that the reduced activation of the Na^+/Ca^{2+} exchanger in the presence of micromolar Ca^{2+} is associated with reduced proteolytic degradation.

Discussion

Recently, Na^+/Ca^{2+} exchangers from arthropods have attracted attention because the exchanger from *Drosophila*, expressed in *Xenopus* oocytes, appears to be catalytically regulated by cytoplasmic Ca^{2+} in an unusual way. Whereas exchangers in mammalian heart, in squid axon, and in barnacle muscle all are catalytically *acti-*

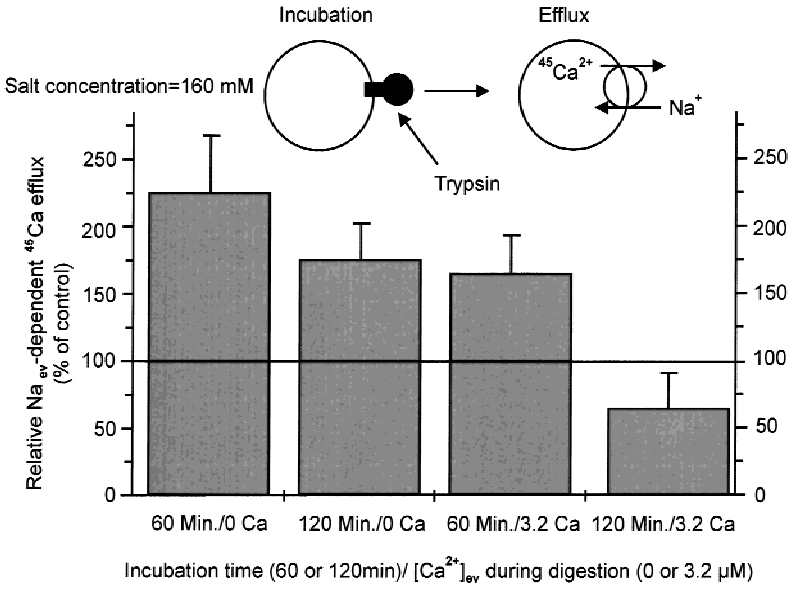

Fig. 7. Effect of trypsin on Na^+/Ca^{2+} exchange activity. The diagram on the top shows the protocol. Aliquots of proteoliposomes were incubated with 0.25 μg/μl trypsin for 60 or 120 min in the virtual absence of Ca_{ev} (two left-hand bars) or in the presence of 3.2 mM Ca^{2+} (two right-hand bars); conditions are comparable to those in diagram in Fig. 6). A 10 sec Na_{ev}-dependent ^{45}Ca efflux was performed in the presence of 10 μM EGTA (i.e., free Ca_{ev} <0.05 μM, as in the experiments described in Fig. 6). Data are given as percent of control ± SE; $n = 4$). The control was defined as the Na_{ev}-dependent $^{45}Ca^{2+}$ efflux measured immediately (<1 min) after addition of the trypsin.

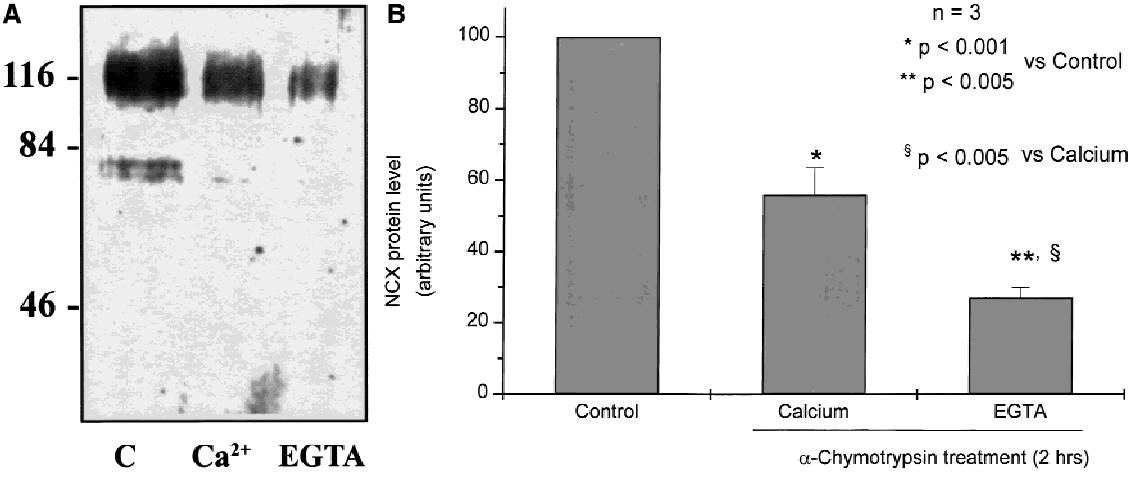

Fig. 8. Effect of limited proteolysis on the intact Na^+/Ca^{2+} exchanger (NCX) protein levels in proteoliposomes (*A*) Western blot analysis of the proteins extracted from control proteoliposomes (lane C), and proteoliposomes subjected to α-chymotrypsin treatment in the presence of 1.9 μM Ca^{2+} (lane Ca^{2+}) and in the presence of 10 μM EGTA without added Ca^{2+} (lane EGTA). The positions of the molecular mass markers are indicated on the left in kilodaltons (kDa). (*B*) Data were normalized to the amount of NCX detected in control samples and are expressed as means ± SD (three independent experiments; statistics refer to a Student's paired *t*-test).

vated by cytoplamic Ca^{2+} [6, 12, 14, 29, 30], the activity of the expressed *Drosophila* exchanger is *reduced* by cytoplasmic Ca^{2+} [19].

Previously, we studied the electrogenic properties of the partially (≈50%) Na^+/Ca^{2+} exchanger from the muscle of another arthropod, the lobster reconstituted into proteoliposomes [11]. Here, we used this preparation to determine whether a cytoplasmic regulatory Ca^{2+} binding site is present and, if so, to compare its properties to those of the exchanger from *Drosophila* and other species.

The lobster muscle exchanger has not been cloned, and its molecular architecture has not been characterized. Nevertheless, this exchanger cross-reacts with antibodies raised against the canine heart exchanger, and has a molecular weight of about 120 kDa (comparable to that of the mammalian cardiac exchanger) [11]. Thus, it seems probable that there is much tertiary as well as primary structural homology between the mammalian cardiac exchanger and the lobster muscle exchanger. This homology likely includes the large cytoplasmic loop between transmembrane segments five and six that is believed to be involved in catalytic regulation of the exchanger activity [22, 26].

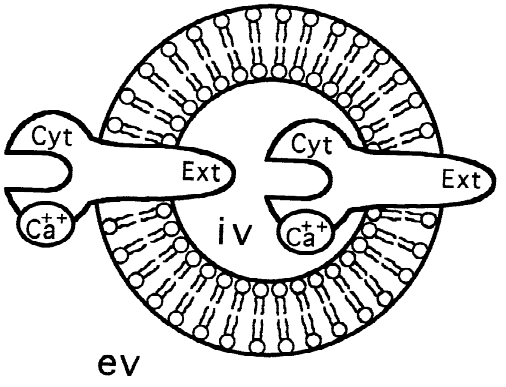

Fig. 9. Schematic representation of proteoliposomes containing randomly orientated Na^+/Ca^{2+} exchanger molecules. Native extracellular domains are indicated as *ext*, cytoplasmic portions are designated as *cyt* and include a Ca^{2+} ion bound to the "catalytic activation" site. To visualize the suffixes used in the text, the intravesicular space is indicated by *iv*, and the extravesicular space by *ev*. Note that in vesicles containing randomly oriented exchanger molecules, cytoplasmic domains or binding sites are exposed on the *ev* surface as well as on the *iv* surface of the proteoliposomes.

Regulation of the Na_{ev}-dependent ^{45}Ca Efflux from Proteoliposomes by Ca_{ev}

To characterize the Na^+/Ca^{2+} exchanger's regulatory high affinity Ca^{2+} binding site ($K_{d(Ca)reg} < 1$ μM in all preparations tested to date [3]), it is essential to gain direct access to the exchanger's cytoplasmic surface. We assumed that the exchanger molecules insert in random orientation in the proteoliposome lipid membranes, so that the cytoplasmic surfaces of ≈50% of the exchanger molecules face the ev medium. A diagram of the proteoliposomes, and the terminology we used to characterize the different orientations and compartments are given in Fig. 9. In all nerve and muscle Na^+/Ca^{2+} exchanger preparations thus far studied (including mammalian cardiac muscle, barnacle muscle, and squid giant axon), extracellular Ca^{2+} binds only to a much lower affinity site ($K_{d(Ca)ext}$ usually > 100 μM) that is directly involved in translocation of the Ca^{2+} [3]. Also, the lobster exchanger exhibits at least two classes of Ca^{2+} binding sites with very different affinities for Ca^{2+} ($K_{d(Ca)cyt}$ = 7–10 μM; $K_{d(Ca)ext}$ >> 40 μM) [11]. These sites correspond to the Ca^{2+} transport sites on the intracellular and extracellular surface, respectively [2, 3, 6, 34]. Accordingly, the exchangers in the proteoliposomes that are oriented with their extracellular surfacees facing the *ev* medium should be distinguishable from those with their cytoplasmic surfaces facing the *ev* medium by the absence of a high affinity Ca^{2+} binding site in the former. Therefore, we studied the influence of $[Ca^{2+}]_{ev}$ on the Na_{ev}-dependent efflux of ^{45}Ca from the proteoliposomes.

Figures 2 and 3 demonstrate that Ca_{ev} activates, with high affinity ($K_{d(Ca)reg}$ = 0.86 μM), the Na_{ev}-dependent ^{45}Ca efflux from the proteoliposomes. These data, as well as the data from the proteolysis experiments (*see below*), are consistent with the view that this Ca^{2+} efflux is mediated by exchanger molecules oriented with their cytoplasmic surfaces facing the *ev* fluid, and that binding of Ca^{2+} to a high affinity site on the cytoplasmic surface *activates* the exchanger. In this respect, the lobster muscle exchanger is similar to the exchangers in mammalian heart, squid giant axon, and barnacle muscle, and is different from the *Drosophila* exchanger expressed in *Xenopus* oocytes.

This Ca^{2+} binding site is distinct from the cytoplasmic Ca^{2+} transport site, as revealed by measurement of the Na_{ev}-independent Ca_{ev}-dependent $^{45}Ca^{2+}$ efflux (Ca^{2+}/Ca^{2+} exchange; Fig. 4). These data ($K_{d(Ca)cyt}$ = 7.6 μM and Hill coefficient, n_H = 1.5) are consistent with measurements of the Ca^{2+} dependence of Na^+/Ca^{2+} exchanger-mediated currents generated by liposomes attached to black lipid membranes [11]. In those experiments the relevant exchanger molecules were also oriented with their cytoplasmic surface facing the *ev* medium; however, current was generated by exchanging Ca_{ev} for Na_{iv} (i.e., the "Ca^{2+} efflux mode" of exchange). Thus, Ca_{ev} needed to bind to the transport as well as to the catalytic Ca^{2+} binding sites; the lower affinity binding to the transport sites ($K_{d(Ca)cyt}$ = 7.6 μM) was then rate limiting. This affinity is consistent with a majority of observations on other species [1, 2, 3, 6, 17]. A Hill coefficient > 1.0 raises the possibility of cooperative action of Ca^{2+} ions, possibly between the catalytic and transport sites, since their dissociation curves overlap.

Loss of Regulatory Control at the Cytoplasmic High Affinity Ca^{2+} Binding Site by Proteolysis

The Na^+/Ca^{2+} exchanger from lobster muscle is also activated by mild proteolysis (Fig. 5). The extent of stimulation is the same as that observed with stimulation by *ev* (cytoplasmic) Ca^{2+}, as described above. Activation by Ca^{2+} and by proteolysis are not additive: proteolytic cleavage not only activates the exchanger, but also presents cytoplasmic Ca^{2+} from regulating exchanger activity in lobster muscle, as in several other species [12, 14, 24, 29]. Low dose trypsin also augments exchange activity, as reported by others [24, 29]. The sites of proteolytic cleavage are not known. α-Chymotrypsin preferably attacks proteins at amino acid sequences with large hydrophobic side chains (Tyr, Phe, Trp), and the large cytoplasmic loop of the *Drosophila* exchanger and the mammalian cardiac exchanger isoforms contain numerous possible cleavage sites.

Binding of Ca^{2+} to the High Affinity Regulatory Site Prevents Activation of the Exchanger by Proteolytic Cleavage

A striking, novel finding is the antagonism, by Ca^{2+}, of the chymotryptic activation (Fig. 6) and proteolytic deg-

radation (Fig. 8) of the Na^+/Ca^{2+} exchanger. α-Chymotrypsin (120 min at free $Ca^{2+} < 0.05$ μM) induced a 2.5-fold activation, whereas incubation for the same period in the presence of 1.9 μM Ca^{2+} induced only a 1.6-fold activation. Concomitantly, about twice the amount of intact (residual) exchanger protein could be detected immunochemically after digestion in the presence of 1.9 μM Ca^{2+} as after digestion in the presence of EGTA. α-Chymotrypsin is not inhibited by micromolar Ca^{2+}, indeed, standard assays include millimolar Ca^{2+} [13]. The reduced proteolysis in the presence of only 1.9 μM Ca^{2+} therefore suggests that, when the exchanger binds Ca^{2+} with high affinity, the cytoplasmic loop folds so as to reduce the accessibility of critical cleavage sites to the proteolytic enzymes is compared to the unbound state.

Low Salt (Hypo-osmotic) Solutions Reduce the Activity of the Lobster Na^+/Ca^{2+} Exchanger

Another novel and unanticipated observation is the influence of the salt concentration on the activity of the lobster exchanger. Most of our early experiments on the lobster preparation were made under ionic conditions that are approximately isosmotic for mammals (about 320 mOsm/l). In some experiments, however, the total salt concentration at the time of vesicle preparation was increased to mimic the osmotic conditions that prevail in marine invertebrates (about 900 mOsm/l). Under these conditions, we observed a surprisingly large increase (3- to 9-fold, in different preparations) in the Ca^{2+} flux mediated by the lobster exchanger without significant change in its sensitivity to proteolysis or to regulation by Ca^{2+}. The increased activity could be due to stabilization of the polypeptide structure by the higher, physiological salt concentration, as well as to changes in the electrostatic microenvironment (e.g., surface charges on the proteoliposomes), as a result of the higher ionic strength. In the high salt environment, the proteoliposomes may be less leaky to Na^+ and Ca^{2+} ions so that, under conditions that favor Na_{iv}-dependent $^{45}Ca^{2+}$ uptake, the driving Na^+ gradient is stable over a longer time, to account for the observed increase in Ca^{2+} load (*see* Results). Furthermore, under $^{45}Ca^{2+}$ efflux conditions, passive Ca^{2+} efflux is also reduced, thereby leaving higher mean $[Ca^{2+}]_{iv}$ for the period of an efflux experiment.

Turnover Rates of the Lobster Na^+/Ca^{2+} Exchanger

Our data can be used to estimate the rate of turnover of the lobster exchanger. According to Fig. 3, the maximum Ca_{ev}-activated, Na_{ev}-dependent Ca^{2+} efflux is about 133 nmol/mg protein × sec at 22–24°C. If about 50% of the protein is Na^+/Ca^{2+} exchanger [12], and about 50% of the exchanger molecules (Mol. Wt. = 120 kDa) are oriented with their cytoplasmic surface facing the ev medium, then 1 mg total protein corresponds to 0.25 mg of exchanger protein, or 2 nmol, with the cytoplasmic side facing out. Thus, the calculated turnover is 133 nmoles Ca^{2+}/sec per 2 nmoles exchanger, or ≅67 sec^{-1}, assuming that 100% of the exchanger molecules are functional. At high (normotonic) salt concentrations, the transport rate is increased by about 6.2-fold, giving a turnover of ≅415 sec^{-1} at 22–24°C. This is probably a low estimate, but seems reasonable compared to published turnover rates of 1000–5000 sec^{-1} at 37°C for the mammalian cardiac Na^+/Ca^{2+} exchanger [3, 4, 15, 20, 27].

Conclusions

The ^{45}Ca flux studies described in this report demonstrate that the lobster muscle Na^+/Ca^{2+} exchanger is regulated by Ca^{2+} at a high affinity ($K_{d(Ca)reg}$ = 0.6 μM) cytoplasmic binding site. This site can be distinguished from the cytoplasmic Ca^{2+} transport site, which has a 10-fold lower affinity for Ca^{2+} ($K_{d(Ca)cyt}$ = 7.6 μM). The regulation by Ca^{2+} can be removed by mild proteolysis with α-chymotrypsin; following proteolysis, the exchanger becomes fully activated in the absence of cytoplasmic Ca^{2+}. Micromolar Ca^{2+} partially protects the exchanger from proteolysis. The activation of the lobster exchanger by cytoplasmic Ca^{2+} appears comparable to that observed for the squid axon, barnacle muscle and mammalian cardiac exchangers, and differs from the downregulation, by Ca^{2+}, of the *Drosophila* exchanger expressed in *Xenopus* oocytes.

The 3- to 9-fold greater Na^+/Ca^{2+} exchange rate observed at high salt (900 mOsm/l) than at low salt (320 mOsm/l) concentrations was unexpected. This might be due to increased exchanger stability, reduced passive loss of Ca^{2+} from the proteoliposomes, and/or an altered electrostatic microenvironment in the liposomal membrane.

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