J. Membrane Biol. 145, 151–164 (1995)

The Journal of
Membrane
Biology

© Springer-Verlag New York Inc. 1995

Electrical Currents Generated by a Partially Purified Na/Ca Exchanger from Lobster Muscle Reconstituted into Liposomes and Adsorbed on Black Lipid Membranes: Activation by Photolysis of Caged Ca^{2+}

A. Eisenrauch,[1] M. Juhaszova,[2] G.C.R. Ellis-Davies,[3] J.H. Kaplan,[3] E. Bamberg,[1] M.P. Blaustein[1,2]

[1]Laboratory of Biophysical Chemistry, Max-Planck-Institute für Biophysik, Kennedyallee 70, D-60596 Frankfurt/M, Germany
[2]Department of Physiology, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, MD 21201
[3]Department of Physiology, University of Pennsylvania School of Medicine, University Walk, Philadelphia, PA 19104 Department of Biochemistry and Molecular Biology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201-3098

Received: 17 August 1994/Revised: 13 January 1995

Abstract. The Na/Ca exchanger from lobster muscle crossreacts specifically with antibodies raised against the dog heart Na/Ca exchanger. Immunoblots of the lobster muscle and mammalian heart exchangers, following SDS-PAGE, indicate that the invertebrate and mammalian exchangers have similar molecular weights: about 120 kDa. The exchanger from lobster muscle was partially purified and functionally reconstituted into asolectin vesicles which were loaded with 160 mM NaCl. ^{45}Ca uptake by these proteoliposomes was promoted by replacing 160 mM NaCl in the external medium with 160 mM KCl to produce an outwardly-directed Na^+ concentration gradient. When the proteoliposomes were adsorbed onto black lipid membranes (BLM), and DM-Nitrophen-Ca^{2+} ("caged Ca^{2+}") was added to the KCl medium, photolytically-evoked Ca^{2+} concentration jumps elicited transient electric currents. These currents corresponded to positive charge exiting from the proteoliposomes, and were consistent with the Na/Ca exchanger-mediated exit of 3 Na^+ in exchange for 1 entering Ca^{2+}. The current was dependent upon the Ca^{2+} concentration jump, the protein integrity, and the outwardly directed Na^+ gradient. KCl-loaded proteoliposomes did not produce any current. Low external Na^+ concentrations augmented the current, whereas Na^+ concentrations >25 mM reduced the current. The dependence of the current on free Ca^{2+} was Michaelis-Menten-like, with half-maximal activation ($K_{M(Ca)}$) at <10 μM Ca^{2+}. Caged Sr^{2+} and Ba^{2+}, but not Mg^{2+}, also supported photolysis-evoked outward current, as did Ni^{2+}, but not Mn^{2+}. However, Mg^{2+} and Mn^{2+} augmented the Ca-dependent current, perhaps by facilitating the adsorption of proteoliposomes to the BLM. The Ca-dependent current was irreversibly blocked by La^{3+} (added as 200 μM DMN-La^{3+}). The results indicate that the properties of the Na/Ca exchanger can be studied with these electrophysiological methods.

Key words: Lobster muscle — Na/Ca exchanger — Proteoliposomes — Planar lipid bilayers — Capacitive coupling — Caged Ca^{2+}

Introduction

The excitable properties of skeletal muscle of arthropods and molluscs differ from those of vertebrate skeletal muscle. In the latter, Na^+ is the main inward current carrier during cell activation (Adrian, Chandler & Hodgkin, 1970; Costantin, 1977) whereas, in the invertebrate muscles, Ca^{2+} is the dominant inward current carrier (Fatt & Ginsborg, 1958; Hagiwara, Chichibu & Naka, 1964; Costantin, 1977). Consequently, invertebrate muscle fibers must possess powerful Ca^{2+} extrusion mechanisms to remove Ca^{2+} from the cells during recovery from activation in order to restore normal Ca^{2+} balance. Two mechanisms that mediate Ca^{2+} extrusion across the sarcolemma are the P-type, calmodulin-sensitive, ATP-driven Ca^{2+} pump (Ca-ATPase; Carafoli & Stauffer, 1994) and the Na/Ca exchanger (Russell & Blaustein, 1974; Ruscak et al., 1987*a*; Blaustein et al., 1991). The exchanger plays a particularly prominent

Correspondence to: A. Eisenrauch

role in Ca^{2+} extrusion from two other types of excitable cells in which excitation is associated with a relatively large influx of Ca^{2+}, namely, vertebrate cardiac muscle (Sheu & Blaustein, 1992) and neurons (Blaustein, 1988). The Na/Ca exchanger in invertebrate muscle fibers also has a large capacity (i.e., maximal velocity) to transport Ca^{2+} (Ruscak et al., 1987*a*; Rasgado-Flores, Santiago & Blaustein, 1989; Blaustein et al., 1991), and likely plays a major role in extruding Ca^{2+} during recovery from activation in these cells. This implies that the Na/Ca exchanger is relatively abundant in the sarcolemma of invertebrate skeletal muscle cells. Therefore, invertebrate muscle would appear to be a good source of exchanger for purification and incorporation into liposomes for kinetic studies.

The exchanger from invertebrate muscle (Rasgado-Flores et al., 1989), like that from the vertebrate heart (Reeves & Hale, 1984), has a coupling ratio (stoichiometry) of 3 Na^+:1 Ca^{2+}, and is therefore electrogenic (Baker et al., 1969; Caroni, Reinlib & Carafoli, 1980; Kimura, Miyamae & Noma, 1987; Caputo, Bezanilla & DiPolo, 1989). Thus, this transport system can be examined with electrophysiological techniques as well as with tracer fluxes and ion-selective probes. Planar bilayer methods, particularly in combination with purified transport proteins incorporated into liposomes, and with the use of caged compounds, provide a powerful alternative to the common electrophysiological techniques for the investigation of electrogenic transport processes. Variants of these methods enable investigations on transient kinetics as well as stationary current experiments, and the methods have previously been applied to light-driven and ATP-driven ion transport systems (Fahr, Läuger & Bamberg, 1981; Fendler et al. 1985; Borlinghaus, Apell & Läuger, 1987; Hartung et al. 1987; Eisenrauch & Bamberg 1990; van der Hijden et al. 1990). These methods are based on the principle of capacitive coupling of proteoliposomes (containing an electrogenic transporter) to a planar bilayer (black) lipid membrane (BLM). The BLM thereby acts as a capacitive electrode that senses the electrogenic events generated by the attached proteoliposomes. The introduction of caged Ca^{2+}, DM-Nitrophen-Ca^{2+} (Kaplan & Ellis-Davies 1988), which can be used to generate Ca^{2+} concentration jumps within a few milliseconds (McCray et al., 1992), enables us to activate, rapidly, Ca^{2+}-dependent electrogenic processes that are driven by a Ca^{2+} concentration gradient (Niggli & Lederer, 1993).

Here we describe the purification of functional Na/Ca exchanger from lobster muscle, and its reconstitution into proteoliposomes. This exchanger crossreacts specifically with antibodies raised against the canine myocardial Na/Ca exchanger. We show that proteoliposomes containing the lobster muscle exchanger, when attached electrostatically to a BLM, can be activated by Ca^{2+} concentration jumps. The resulting capacitive currents are consistent with an outward movement of 3 Na^+ in exchange for 1 Ca^{2+} that enters the proteoliposomes via the Na/Ca exchanger. This is the first time that a Na^+ gradient-coupled transport system has been studied with these planar bilayer methods. The results indicate that these methods may be suitable for studying some of the molecular events associated with the translocation of Na^+ and Ca^{2+} via the Na/Ca exchanger.

Materials and Methods

Preparation of Lobster Muscle Plasma Membrane

Plasma membrane was prepared from the tail and claw muscles of the lobster, *Homarus americanus*, by a modification of the method of Ruscak and colleagues (1987*a*). Excised striated muscle was minced and suspended in ice-cold medium containing (in mM): sucrose, 300; EGTA, 5; Tris-HCl, 20, at pH 7.4. About 70 gm of tissue was obtained from one 500 gm lobster. The tissue-to-medium ratio was 1:5 (w/v). The tissue was homogenized with six 30 sec strokes in a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY). This and all subsequent preparative steps were carried out at 4°C. The homogenate was centrifuged for 20 min at 12,000 × *g* in an SS-34 rotor (Sorvall RC-5B centrifuge; DuPont, Wilmington, DE). The supernatant was decanted, and the sediment was resuspended in fresh medium (1:3, w/v), rehomogenized, and recentrifuged, as above. The pooled supernatants were filtered through several layers of cheesecloth and subsequently centrifuged at 100,000 × *g* in a Beckman LP-50 ultracentrifuge (45 Ti rotor) for 1 hr. The sediment was stirred in 100 ml of 600 mM KCl, 20 mM Tris-HCl pH 7.4 and, after 1 hr incubation (4°C), the suspension was centrifuged at 100,000 × *g* for 1 hr. The sediment was suspended in 45% sucrose and layered on the bottom of a discontinuous sucrose density gradient consisting of 11, 26, 29, 34, and 36% sucrose in 20 mM Tris-HCl pH 7.4. The gradient was centrifuged at 100,000 × *g* in a Beckman SW 28 ultracentrifuge rotor for 16 hr. The 26% sucrose band, containing the sarcolemmal fraction, was diluted (1:5, v/v) with a 5 mM Tris-HCl, pH 7.4, and centrifuged for 1 hr at 100,000 × *g*. The resulting pellet (= "muscle membranes") was stirred in 160 mM NaCl, 20 mM Tris-HCl, pH 7.6 (about 50 mg membrane protein/10 ml). Protein concentrations were determined by the BCA protein assay reagent (Pierce, Rockford, IL), using bovine serum albumin (BSA) as a standard. The yield was about 50 mg of muscle membrane protein from 70 gm of muscle. The membranes were stored at −80°C.

Purification of the Lobster Na/Ca Exchanger

The Na/Ca exchanger was partially purified as described (Durkin et al., 1991*a*) with slight modification. Lobster muscle membranes (about 50 mg membrane protein) were first subjected to alkaline extraction (Philipson et al., 1987; 1988). The membranes were diluted about 10-fold with ice-cold 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (Caps) buffer, pH 12 (with NaOH). After 10 min incubation on ice, the membranes were centrifuged at 120,000 × *g* for 30 min. The supernatant was discarded and the alkali-extracted pellet was resuspended in 40 ml solubilization buffer containing: 2.5% cholate (Fluka Chemical Corp., Ronkonkoma, NY), 2 mg/ml asolectin (Associated Concentrates, Woodside, NY), 100 mM NaCl, and 20 mM Tris-HCl, pH 7.5. After 1 hr of incubation (4°C) with continuous stirring, the residual insoluble residue was pelleted by a 1 hr centrifugation at 200,000 × *g*. Asolectin consists of approximately equal proportions of lecithin, ceph-

alin and phosphatidylinositol along with minor amounts of other phospholipids. The asolectin was important for preserving exchanger activity during subsequent chromatography. The supernatant was applied to an Affi-Gel 102 (Bio-Rad, Melville, NY) column, (bed volume = 30 ml, equilibrated with solubilization buffer), and bound material (including the exchanger protein) was eluted with 800 mM NaCl in solubilization buffer.

Reconstitution of the Na/Ca Exchanger into Proteoliposomes

Protein eluted from the column was reconstituted by addition of 40 mg/ml asolectin to the eluate (*cf.* Ruscak et al., 1987*b*). The asolectin was solubilized by homogenization of the material with a Teflon-glass homogenizer, and then incubated on ice for 30 min. Insoluble residue was pelleted by centrifugation at 200,000 × *g* in a Beckman 70.1 Ti rotor. The supernatant was collected by aspiration and was transferred to a Spectra/Por dialysis membrane (Spectrum, Houston, TX). The detergent was removed by overnight dialysis against a 1000-fold volume excess of dialysis buffer containing (in mM): 800 NaCl, 1 2-mercaptoethanol, and 20 Tris-HCl, pH 7.5. Further formation of proteoliposomes was achieved by dilution of the dialysate 1:6 (v/v) with dialysis buffer. Reconstituted proteoliposomes were collected by centrifugation at 200,000 × *g* for 3 hr, and were resuspended (50 μg protein/ml) in 160 mM NaCl, 20 mM Tris-HCl, pH 7.5. The proteoliposomes were stored at −80°C.

Determination of the Na/Ca Exchange Activity

Na/Ca exchange activity was determined as the Na^+ gradient ($[Na^+]_i \gg [Na^+]_o$) dependent $^{45}Ca^{2+}$ uptake (Reeves & Sutko, 1983). Aliquots (5 μl) of internal Na^+-loaded proteoliposomes (or membranes) were transferred into 250 μl of KCl (or NaCl) buffer containing (in mM): 160 KCl (or 160 NaCl), 40 μM $CaCl_2$ with 0.3 μCi $^{45}CaCl_2$, and Tris-HCl, pH 7.5. Following a 1 sec incubation (22°–24°), the samples were rapidly filtered through a mini-column (1 cm × 1 cm) of CM-Sepharose (Bio-Rad, Melville, NY) to remove extracellular tracer (Ruscak et al., 1987*b*). The CM-Sepharose (1 g/25 ml) was equilibrated with either the KCl or the NaCl buffer. The eluates from the columns were collected in scintillation vials. An aliquot (8 ml) of Complete Counting Cocktail (Research Product International Corp., Mount Prospect, IL) was added to each vial, and the radioactivity was measured in a Rack-Beta (LKB Wallac, Turku, Finland) liquid scintillation counter. The Na^+ gradient-dependent, (Na/Ca exchanger-mediated) Ca^{2+} uptake was calculated as the Ca^{2+} uptake from the KCl buffer minus the Ca^{2+} uptake from the NaCl buffer.

The protein concentration in the proteoliposomes was determined by the method of Kaplan & Pedersen (1985). This method enabled us to measure microgram quantities of protein in the presence of milligram levels of lipid.

Protein Electrophoresis and Silver Staining

Samples of vesicles/reconstituted proteoliposomes were obtained at various stages of the purification procedure to estimate the extent of Na/Ca exchanger purification. Prior to electrophoresis, the samples were precipitated with 12.5% trichloracetic acid and then centrifuged at 10,000 × *g* for 30 min. The lipids were then extracted from the pelleted vesicles/proteoliposomes with three 2 ml aliquots of a warm ethanol-diethyl ether mixture (1:1, v/v). The proteins were then solubilized in sodium dodecyl sulfate (SDS) sample buffer [containing 12.5 mM Tris (pH 6.8), 0.5% SDS, 10% glycerol and 1% 2-mercaptoethanol] for 7 min at 100°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemli, 1970). The gel was calibrated with prestained protein molecular mass markers (Bio-Rad, Richmond, CA). The electrophoretically separated proteins were silver stained as described (Oakley et al., 1980).

Immunoblotting

The membrane proteins from rat heart (Ambesi et al., 1991*b*) and lobster muscle were separated by SDS-PAGE as described above. Separated proteins were transferred to a Nitro-Bind nitrocellulose membrane (MSI, Westboro, MA) as described (Towbin & Gordon, 1984). The extent of transfer was checked by Ponceau-S staining. Membranes were blocked with 5% nonfat dry milk in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (137 mM NaCl; 20 mM Tris, pH 7.6) with 0.1% Tween 20 (TBST) for 6 hr at room temperature. The blots were then incubated overnight at room temperature with either preimmune serum or polyclonal antibodies (1:500 dilution in TBST) raised against the purified dog cardiac sarcolemmal Na/Ca exchanger (generously provided by Dr. G.E. Lindenmayer, Med. Univ. S. Carolina). The membranes were washed and incubated for 2 hr in TBST containing 0.25 Ci/ml 125I-labeled protein A (Amersham, Arlington Heights, IL), washed again, and exposed to Kodak (Rochester, NY) X-Omat film.

Planar Lipid Bilayer Experiments

The setup of the experiments, and the main principles of activation of the Na/Ca exchanger and of the current measurement are summarized schematically in Fig. 1. BLM were prepared according to the method of Müller et al. (1962) using a lipid mixture consisting of 1.5% (w/v) diphytanoyl-phosphatidylcholine: octadecylamine (60:1, w/w) in decane. The lipid mixture was spread over a 0.5 mm diameter hole that separated two compartments of a teflon cuvette. The development of the BLM was controlled by observation of the membrane capacitance. These membranes have a net positive charge under the conditions used here; this facilitates the attachment of negatively charged proteoliposomes (Dancshazy & Karvaly, 1976; Hermann & Rayfeld, 1976). For these experiments, thawed aliquots of proteoliposome suspensions were sonicated for 10–15 sec (Bransonic 12 bath sonicator, Branson Sonic Power Co., Danbury CT).

Electrolyte solutions (containing DM-Nitrophen-Ca^{2+}, etc.) and proteoliposomes were added to the trans compartment (right-hand compartment in Fig. 1*A*). Ca^{2+} and the other divalent cations (Me^{2+}) used were always added together with the caging compound DM-nitrophen (DMN). DMN was always added in excess over the cation (usually at a DMN:Me^{2+} ratio of 3:2; *see* Results) in order to chelate all divalent cations between flashes. Aliquots of the proteoliposome suspension (0.8–6.0 μg protein) were then added to the trans compartment, and an equal volume of fluid was removed from this compartment. The compartment contents were stirred for at least 45 min in order to allow time for the proteoliposomes to attach to the BLM. Divalent cation transients were initiated by 250 msec UV light flashes from an Osram HBO 200 W mercury lamp. The data collection was started when vesicle attachment appeared to reach a stable level and the peak current amplitude became constant. Since the illuminated part of the cuvette was only about 1% of the total cuvette volume, effects due to the loss of DMN were minimal. To eliminate concentration gradients in the solutions (due to sample additions and photolysis of DMN), the fluids in both compartments were continuously stirred between flashes. The usual frequency of flashing was one flash every 10 min.

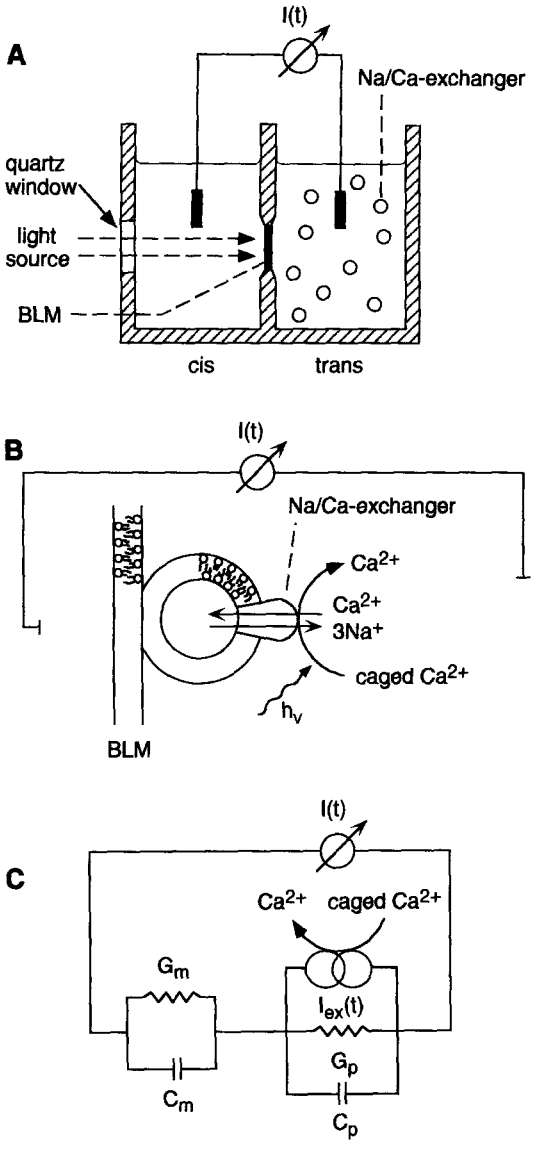

Fig. 1. Principle of the method. (*A*) Setup of the teflon cuvette. The arrows from the light source point to the BLM which covers the septum between the two compartments. The "trans" compartment is on the right. The sketch also shows the current measuring circuit and the light path. (*B*) Arrangement of the BLM with attached proteoliposomes. The activation of the Na/Ca exchanger by photolysis of caged Ca^{2+} is diagrammed. (*C*) Equivalent circuit of the BLM and the attached proteoliposomes. G_m and G_p are the ohmic conductances of the BLM and the proteoliposome membrane, respectively. C_m and C_p are the respective capacitances. $I(t)$ represents the measured Na^+ and Ca^{2+} gradient driven current. As discussed in detail elsewhere (Bamberg et al. 1979; Fahr et al. 1981), under the assumption that the intrinsic time constants of the transporter are fast compared to the time constants of the measuring system, the observed time-dependent current is related to the current produced by the electrogenic transporter according to the relationship (Bamberg et al. 1979):

$$I(t) = \left(\frac{C_m}{C_m + C_p}\right) \cdot I_{ex,o} \cdot \exp\left(\frac{t}{\tau}\right)$$

$I_{ex,O}$ is the true current produced by the electrogenic transporter, τ is an intrinsic time constant of the transporter. A possible time-independent (stationary) current term could be omitted, since the conductance of the black lipid membrane is negligible in the absence of ionophores. The amplitude is proportional to the original exchanger current and is attenuated by the capacitive factor. The time resolution is limited by the rise time of the amplifier (here 10 msec; 10–90%), and by the effective time constant of Ca^{2+} release, which includes the relatively slow opening time (5–10 msec) of the shutter.

Materials

DMN was synthesized according to published protocols (Ellis-Davies & Kaplan 1988). L-α-diphytanoylphosphatidylcholine was purchased from Avanti Polar Lipids, Birmingham, AL. Monensin, bovine pancreatic trypsin (type I), and soybean trypsin inhibitor (type I-S) were from Sigma. All other chemicals were analytical grade.

The currents were amplified 10^9-fold and converted to voltage signals by a current amplifier/converter (Model 1211, Ithaco, Leverkusen, FRG). The design of our measuring system led to the convention that positive charge entering the trans compartment was recorded as current responses of negative sign. The data were monitored on a digital storage oscilloscope (Nicolet, Offenbach, FRG) and stored in a computer using a custom-designed data acquisition program. The preparation of most of the figures as well as the fits to the data of the figures 6 and 7 were performed by use of the program Sigma Plot 5.0 (Jandel Scientific, Erkrath, FRG).

The effectiveness of release of Ca^{2+} from DMN-Ca^{2+} was estimated by measuring the free Ca^{2+} concentration after one or two UV light flashes in control experiments. For this purpose, DMN and $CaCl_2$ were added at equal concentrations (usually 300 μM each) to exclude rechelation of the released Ca^{2+}. The amount of Ca^{2+} detected spectroscopically by the change in arsenazo III absorbance was then equal to the amount of DMN photolyzed by the UV light. The response of arsenazo III to Ca^{2+} was calibrated with solutions of known free Ca^{2+} concentration (EGTA buffered) on the basis of the absorption difference at 650–730 nm (Cook & Kaupp, 1988).

Results

Identification and Partial Purification of the Na/Ca Exchanger from Lobster Muscle Membranes

Figure 2 shows that membrane vesicles from lobster muscle exhibit Na^+ gradient-dependent Ca^{2+} uptake. The Na-loaded vesicles accumulated, on average, 5.1 ± 1.0 nmoles Ca^{2+}/mg protein when incubated in KCl buffer for 1 sec, and only 2.0 ± 0.3 nmoles Ca^{2+}/mg, when incubated in NaCl buffer. The difference, which corresponds to the specific activity of the Na/Ca exchange component (ΔNa/Ca) was 3.1 ± 1.0 nmoles Ca^{2+}/mg ($P < 0.05$, n = 6 preparations). When the Na/Ca exchanger was reconstituted into the asolectin proteoliposomes without purification, a partial stimulation of the exchange activity (about 7-fold) was observed. Others (e.g., Hale et al., 1984; Philipson et al., 1987) have reported that reconstitution, *per se*, activates the ex-

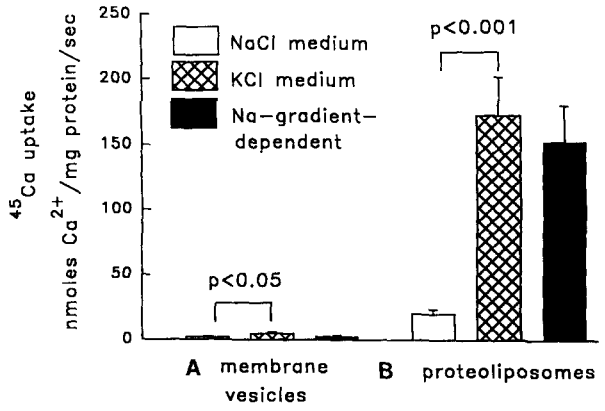

Fig. 2. Specific activities of Na/Ca exchanger in native lobster muscle plasmalemmal vesicles (*A*) and in asolectin proteoliposomes containing purified and reconstituted lobster muscle exchanger (*B*). Vesicles or proteoliposomes loaded with 160 mM NaCl buffer were diluted into NaCl buffer (–Na gradient; open bars) or KCl buffer (+Na gradient; cross-hatched bars). The difference between the Ca^{2+} uptake from these two buffers (delta Na/Ca; solid bars) was taken as the Na/Ca exchange activity.

changer. We found that further purification resulted in a 59-fold increase in exchanger activity in the proteoliposomes (Na/Ca = 186 ± 29 nmoles Ca^{2+}/mg, $P < 0.001$, n = 10, *see* Fig. 2). This specific activity is comparable to that reported for partially purified canine cardiac Na/Ca exchanger (Durkin et al., 1991*a*).

Evidence that the Na/Ca exchanger from lobster muscle is similar in molecular weight and immunological crossreactivity to canine cardiac Na/Ca exchanger is presented in Figs. 3 and 4. SDS-PAGE (Fig. 3) of lobster muscle membranes (lane *A*), alkali-extracted membranes (lane *B; see* Methods), and protein from the proteoliposomes containing partially purified exchanger (lane *C*) demonstrate that most of the original membrane proteins (*A*) are no longer present in the partially purified material, which contains two major bands, one at 120 kDa, and a doublet at about 70 kDa (*C*). These bands crossreact specifically (Fig. 4, lane *B*) with rabbit polyclonal antiserum raised against the dog heart Na/Ca exchanger. The exchangers in rat and dog heart are highly homologous (Low, Kasir & Rahamimoff, 1993), and Fig. 4, lane *A* shows the antiserum crossreactivity with rat heart sarcolemma (we obtained similar results with dog heart sarcolemma; Goldman et al., 1994). Preimmune serum exhibited no crossreactivity with these proteins (*not shown*). Note that the mammalian heart exchanger has an apparent molecular weight (of the glycosylated molecule) of about 120 kDa; the 70 kDa band appears to be a proteolytic fragment of the exchanger (Nicoll, Longoni & Philipson, 1990; Durkin et al., 1991*a*).

In sum, the results in Figs. 2–4 show that the exchanger from lobster "skeletal muscle" is similar to that present in the mammalian heart in terms of its physiological activity, molecular weight, and immunological

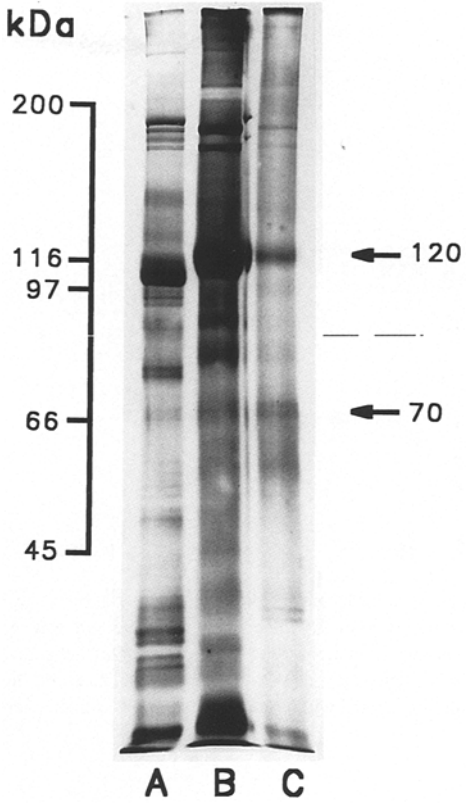

Fig. 3. SDS-PAGE of native membranes and partially purified Na/Ca exchanger. Lane *A:* Native lobster muscle sarcolemmal membranes (10 μg of protein). Lane *B:* Membrane sample following alkaline extraction (200 μl of proteoliposome suspension). Lane *C:* Partially purified Na/Ca exchanger (200 μl of proteoliposome suspension with reconstituted exchanger after partial purification; this is the material that was used for the electrophysiological studies. Prior to SDS-Page the samples in lanes *B* and *C*, which had been reconstituted with asolectin, were extracted with diethyl ether/ethanol (1:1) to remove the lipids. In this representative experiment the total protein content was reduced about 6.5-fold from "*B*" to "*C*", but the specific activity increased from 19.9 nmoles Ca^{2+}/mg (*B*) to 170.9 nmoles Ca^{2+}/mg protein (*C*).

crossreactivity. The proteoliposomes containing the partially purified lobster Na/Ca exchanger were therefore used for electrophysiological studies in all the experiments described below. We did not employ more purified preparations because the partially purified material used here (approximately 30–50% pure) provided a good yield of material (about 100 μg of partially purified protein from 70 gm of muscle), whereas further purification improved the specific activity but was associated with a large loss of total activity.

The Use of DM-Nitrophen in Planar Bilayer Experiments

DMN, when applied together with a divalent cation, at concentrations between 200 and 700 μM, produces a

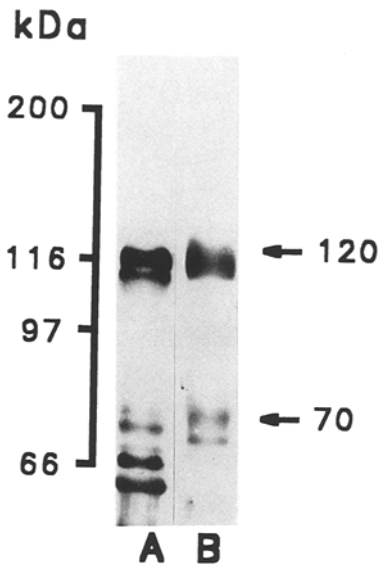

Fig. 4. Immunoblot of rat heart plasmalemmal proteins (lane *A;* 5 μg) and lobster plasmalemmal proteins (lane *B;* 20 μg). The blot was incubated with antiserum raised against the dog heart Na/Ca exchanger. Positions of the molecular mass markers (in kDa) are indicated on the left. The control blot, incubated with preimmune serum, was blank, and is not shown.

small artifact (usually 1–2 pA) of negative sign during 250 msec of illumination with UV light from a mercury lamp (Fig. 5*A*). The addition of protein free asolectin liposomes, as well as the addition of asolectin proteoliposomes containing Na,K-ATPase (a transport protein that does not bind Ca^{2+} specifically) reconstituted in the same way as the Na/Ca exchanger, quenched these nonspecific currents, sometimes to zero. In some preliminary experiments in which the DMN concentration exceeded 1 mM, larger artifacts with variable multiphasic shapes were observed. Therefore, we restricted the DMN concentration in all experiments to a maximum of 700 μM to avoid these large artifacts.

Ca^{2+}-Dependent Currents Generated by the Na/Ca Exchanger

Proteoliposomes containing the partially purified Na/Ca exchanger from lobster muscle, loaded with 160 mM NaCl, were added to the trans compartment (Fig. 1), where they adsorbed to the positively changed BLM. Caged Ca^{2+} was added to the same compartment. A 250 msec UV light flash was used to release Ca^{2+} from the DMN-Ca^{2+} in order to induce a concentration jump of Ca^{2+} in the solution and to activate, simultaneously, a large number of exchanger molecules. All experiments were performed at room temperature (about 20–24°C). Fig. 5*A* shows the small artifact produced by the release of Ca^{2+} from DMN-Ca^{2+} in the absence of proteoliposomes. Upon addition of proteoliposomes much larger currents, up to 300–350 pA in a few instances, were observed after the proteoliposomes were given time to attach to the BLM (Fig. 5*B* shows an example). In all of the experiments discussed below, the quantitative data refer to the peak current amplitude evoked by the light flashes. This current was dependent upon the proteoliposome concentration (not shown). Nevertheless, the current amplitudes varied considerably from experiment to experiment. This variation may, in part, be caused by variation in the exchanger density in the proteoliposomes from different preparations and, in part, by variable attachment of the proteoliposomes to the bilayer. In individual experiments, however, the peak current usually

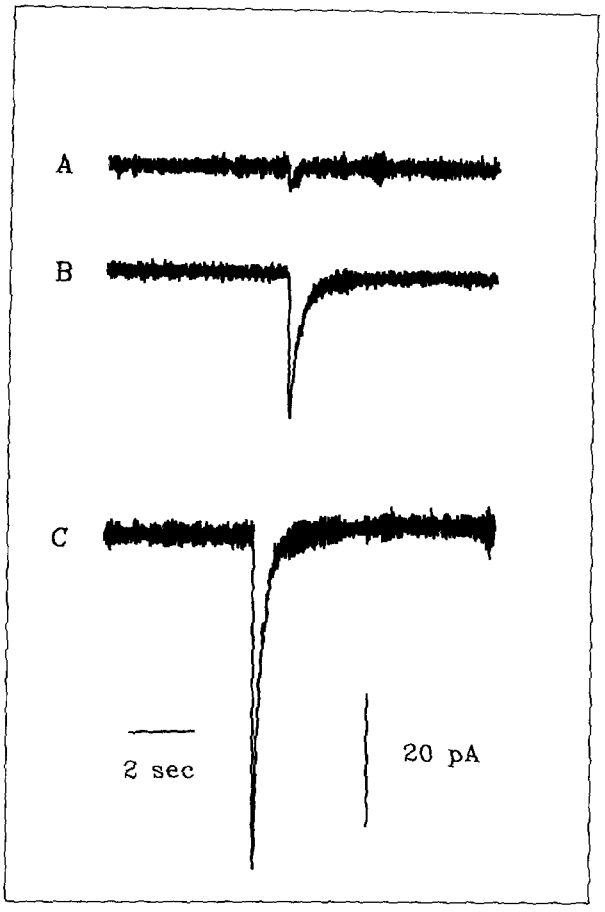

Fig. 5. Representative current responses evoked by photolysis of DMN under different conditions. The cuvette, containing KCl buffer with (trans compartment, only) 300 μM DMN/200 μM $CaCl_2$, was illuminated with UV light for 250 msec. Record *A:* No proteoliposomes present. Artifact due to photolysis of DMN. Peak response amplitude = −1.5 pA (*see* text regarding sign convention); the duration of the response equaled the duration of the illumination. Record *B:* Light-evoked current response after addition, to trans compartment, of proteoliposomes containing 6.0 μg of partially purified Na/Ca exchanger. Record *C:* Addition of 300 μM DMN + 200 μM $MgCl_2$ further increased the light-evoked current. The three records are taken from the same experiment.

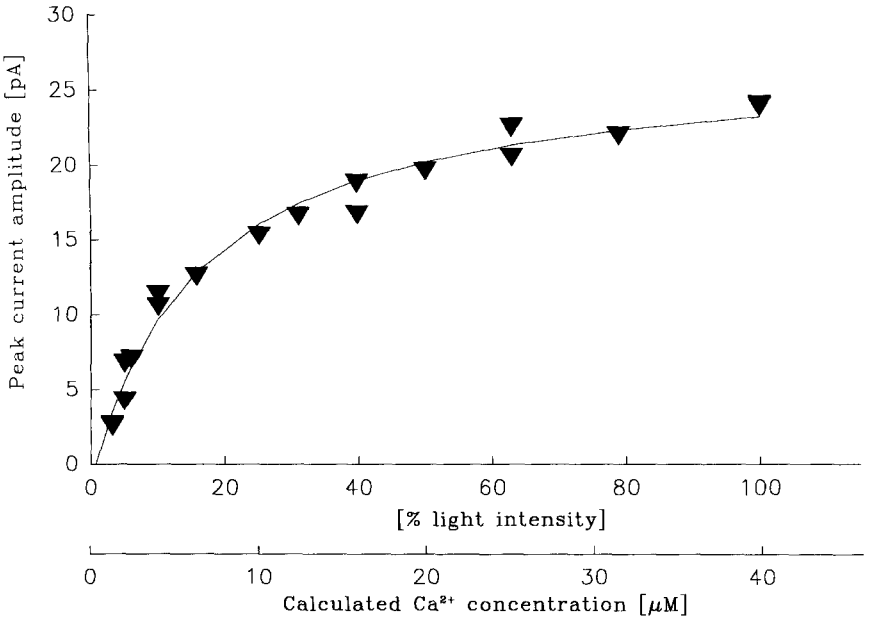

Fig. 6. Relationship between peak light-evoked current and relative light intensity (and Ca^{2+} concentration). Medium = KCl buffer with (trans compartment only) 600/400 μM DMN/Ca and 3 μg proteoliposome protein. The release of different amounts of Ca^{2+} from DMN was controlled by varying the UV transmittance with UV filters. The solid curve was fitted to the data using the Michaelis-Menten equation with the assumption that 100% transmittance corresponds to a Ca^{2+} concentration of 40 μM (i.e., about 10% of the DMN-Ca^{2+} in the light path was photolyzed in a single flash; *see* text). Accordingly, the calculated values for maximum Ca^{2+}-dependent current (I_{max}) and Ca^{2+} concentration for half-maximal activation of the current ($K_{M(Ca)}$) were, respectively 27 pA and 7 μM.

rose to a steady level after several flashes, over the course of 45–60 min, and then tended to remain relatively constant for at least 1–2 hr in the absence of changes in the experimental conditions (e.g., *see below*). As noted above, according to the sign convention used here, the negative sign of the current corresponds to positive charge moving out of the proteoliposomes. This was confirmed by comparison with the current evoked by the release of ATP from "caged ATP" when we employed proteoliposomes containing the mammalian skeletal muscle sarcoplasmic reticulum Ca^{2+} pump. In the latter case, currents of positive sign were observed, indicating that positive charge was moving from the trans compartment into the vesicles, as expected for the uncompensated net uptake of Ca^{2+} (*not shown*).

Addition of excess EGTA, to chelate all of the Ca^{2+} released by the photolysis of DMN-Ca^{2+}, abolished the current (*not shown*). The current was also abolished by the addition of excess Ca^{2+}, which raised the Ca^{2+} concentration in the medium and prevented the photolysis of DMN-Ca^{2+} from evoking a significant Ca^{2+} concentration jump (*not shown*).

Effect of the Ca^{2+} Concentration on the Light-Evoked Response

In order to test the dependence of the exchanger currents on the Ca^{2+} concentration, we varied the light intensities of the UV illumination by using UV filters with different transmittance. Figure 6 shows the dependence of the current amplitudes on the light intensity, which reflects the Ca^{2+} concentration dependence. The rectangular hyperbolic shape of this relationship is clearly Michaelis-Menten like. The parameters giving the best fit to the data (solid line in Fig. 6) were: I_{max} (maximal peak current) = 27 pA and $K_{1/2}$ (relative light intensity for half-maximal response) = 16.5%. The curve extrapolated to "0" response at 0.7% light intensity, presumably because of the rapid rebinding of released Ca^{2+} due to excess DMN. As described in Materials and Methods, we were able to estimate the amount of photolyzed DMN per flash by measuring (in calibration experiments) the amount of Ca^{2+} released, using arsenazo III as a Ca^{2+} indicator. Based on our determination that 10% of the DMN in the light path is photolyzed by a UV flash under the conditions employed here, a maximum Ca^{2+} concentration step of about 40 μM was attainable in the experiment of Fig. 6 (with DMN:Ca^{2+} = 600 μM:400 μM). Thus, the estimated $K_{M(Ca)}$ (Ca^{2+} concentration for half-maximal activation) is about 7 μM.

Effects of Other Divalent Cations (Me^{2+}) on the Light-Evoked Current

Currents of the same (negative) sign were also observed following the release of Sr^{2+} and Ba^{2+}, when these cations were added together with the caging compound, in place of Ca^{2+}, at a ratio of 300 μM DMN: 200 μM Me^{2+} (Table). In the absence of Ca^{2+}, photolysis of caged Mg^{2+} did not induce significant current. However, the addition of caged Mg^{2+} in the presence of caged Ca^{2+} was usually associated with a large increase in the current evoked by photolysis of the DMN (Fig. 5*C* shows an example; *also see* the Table). The Mg^{2+}-induced increase in the Ca^{2+}-dependent current may have been due, at least in part, to increased adsorption of proteolipo-

Table. The ability of various caged divalent cations to generate current when released by photolysis of DMN

Ion	Caged ions*	
	300 μM DMN/ 200 μM ME^{2+}	600 μM DMN/ 200 μM Me^{2+}/ 200 μM Ca^{2+}
	Current (pA)	Current (pA)
Ba^{2+}	−7.6	−45.2
Sr^{2+}	−5.6	−21.0
Ni^{2+}	−29.6	−30.6
Mn^{2+}	0	−138.4
Mg^{2+}	0	−134.2

* All experiments were performed with KCl buffer in the cuvette. The trans compartment contained 0.8 μg of proteoliposome protein, 300 μM DMN and 200 μM of the $MeCl_2$ salt of the ion shown in the left-hand column. Samples from the same proteoliposome preparation were used for all of the studies in this table. The middle column shows the currents generated with the test divalent cation, alone; the right-hand column shows the current generated after the addition of DMN/Ca 300/200 μM.

somes to the BLM. When DMN-Ca^{2+} was added after DMN-Mg^{2+}, there was usually a rapid, large increase in light-evoked current, indicating that many proteoliposomes were already adsorbed to the BLM. In the absence of DMN-Mg^{2+}, on the other hand, the Ca^{2+}-dependent increase in current occurred slowly. Taken together, these data indicate that the negative current is dependent upon a concentration jump due to the release of a suitable alkaline earth ion (but not Mg^{2+}) into the trans compartment. This is consistent with tracer flux evidence that Sr^{2+} and Ba^{2+}, but not Mg^{2+}, can be transported by the Na/Ca exchanger (Blaustein, 1977).

We also tested the effects of Mn^{2+} and Ni^{2+}. No light-evoked current was detected when caged Mn^{2+} was used (Table). However, this cation, like Mg^{2+}, augmented the Ca^{2+}-dependent light response (Table). In contrast, low concentrations of Ni^{2+} (added as 300 μM DMN:200 μM Ni^{2+}) supported a light-evoked current, but had negligible effect on the Ca^{2+}-dependent current (Table).

The Light-Evoked Current is Reduced by Proteolysis

Figure 7 illustrates the effect of 10,500 U of bovine pancreatic trypsin on the current amplitudes. In three experiments, the current amplitude was reduced to 42 ± 10% of the original amplitude after 60 min of incubation with trypsin (at room temp). When the same amount of trypsin was added in the presence of an excess of soybean trypsin inhibitor, the inhibitory effect of the trypsin was abolished (Fig. 7, squares). These data imply that the Ca^{2+}-dependent current is mediated by the intact Na/Ca exchanger protein present in the proteoliposomes; time-dependent proteolytic cleavage caused a progressive loss of activity. The linear decline of the current amplitude in this semilog plot (Fig. 7, triangles) is consistent with a first-order enzymatic hydrolysis of the exchanger.

Effects of Monovalent Ion Concentrations on the Ca^{2+}-Dependent Current

The requirement for Na^+ on the opposite side of the membrane from Ca^{2+} is illustrated in Fig. 8. The Ca^{2+} concentration jump-evoked current was reduced when the Na/H exchanging electroneutral ionophore monensin was added to the distal compartment to reduce the Na^+ concentration (normally 160 mM) in the proteoliposomes. Under the conditions employed here, with NaCl buffer in the proteoliposomes and KCl buffer in the bulk solution, the monensin actually exchanges K^+ for Na^+, so that no H^+ gradient is built up. The addition of the proteoliposomes introduces sufficient Na^+ to give a final Na^+ concentration of 5 mM in the cuvette. If this Na^+ is equally distributed between the interior and exterior of the vesicles after monensin treatment, there could be residual Ca^{2+}-activated, internal Na^+-dependent current, as demonstrated with bovine cardiac Na/Ca exchanger (Durkin et al., 1991*b*). The fact that there is a residual current (Fig. 8), however, also raises the possibility that the Na^+ gradient across the vesicle membrane is not completely dissipated by 5 μM monensin (Fontana et al., 1995). This view is further supported by the known inhibition of monensin mediated Na^+ fluxes at high (>150 mM) Na^+ concentrations (Riddell & Hayer, 1985).

When Na^+-free, K^+-loaded proteoliposomes were used, no current response to the Ca^{2+} concentration jump was detected (*not shown*). When KCl was added to an alkali metal ion-free medium (KCl in the buffer was replaced by isosmotic Tris-HCl), there was no significant effect on the Ca^{2+} concentration jump-evoked current (Fig. 9, circles). In contrast, addition of NaCl under similar conditions caused a concentration-dependent decline in the current (Fig. 9, triangles). These experiments all indicate that the current evoked by the Ca^{2+} concentration jump is dependent upon intravesicular Na^+, and is inhibited by external Na^+ but not by external K^+.

Figure 10 illustrates the activating effect of a low concentration of external Na^+ in KCl buffer. At concentrations below about 30 mM, Na^+ augmented the Ca^{2+}-dependent current. In three experiments, 25 mM NaCl increased the current amplitude by an average of 35 ± 13%. When the NaCl concentration exceeded 30 mM, however, Na^+ acted as an inhibitor (Fig. 10). This effect was not due to the increased osmolarity of the medium because the addition of KCl (Fig. 11) or sucrose (*not shown*) did not exhibit these effects. In fact, the addition

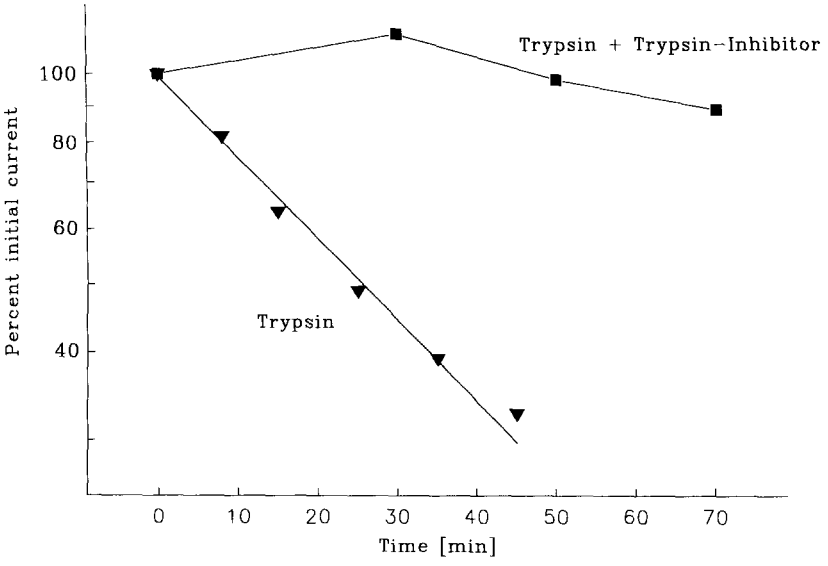

Fig. 7. Effect of trypsin on the light-evoked currents. Medium = KCl buffer containing (trans compartment, only) 600/200/200 μM DMN/Ca/Mg. The data are normalized to the stable peak amplitude immediately before the addition of trypsin (100%). In the experiment represented by the squares, the trans compartment (containing the proteoliposomes) also contained 1 mg soybean trypsin inhibitor (type I-S), which should inhibit approx. 30,000 U of trypsin. In both experiments 1 mg (10,500 U) bovine pancreatic trypsin (type I) was added to the trans compartment at time = "0". The proteoliposome protein concentration was 1 μg for the data represented by the triangles, and 3 μg for the data represented by the squares.

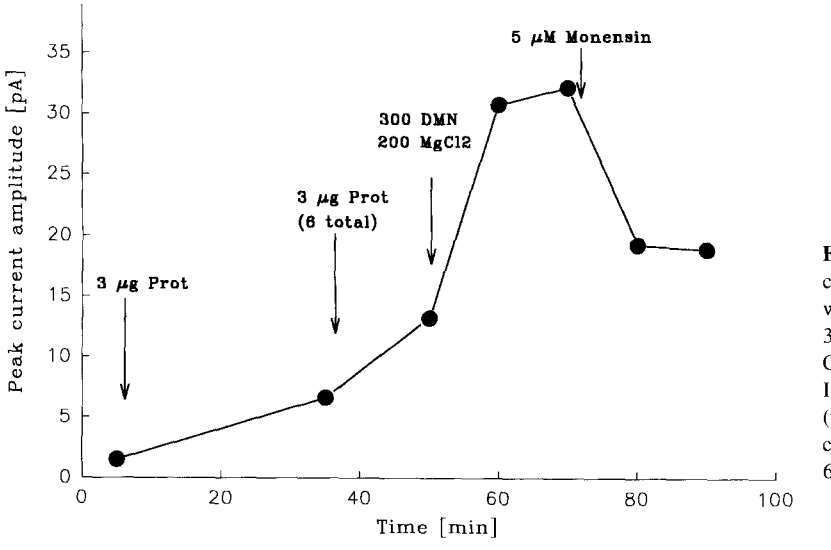

Fig. 8. Effect of monensin on the light-evoked current. Medium = KCl buffer. The experiment was started at time = "0", with the addition of 300/200 μM DMN/Ca to the trans compartment. Other additions are indicated on the figure. Immediately before the addition of 5 μM monensin (final concentration), the trans compartment contained (final concentrations, in μM) 600/200/200 DMN/Ca/Mg.

of KCl to an external solution containing 160 mM N-methylglucamine and 95 mM NaCl slightly increased the peak current amplitude (Fig. 11). The latter result may indicate that there is some cooperativity between the effects of Na^+ and K^+ (*cf.* Fontana et al., 1994); in addition, this figure shows that under our conditions alterations of the signal due to osmotic effects were negligible.

Inhibition of the Ca^{2+}-dependent Response by Lanthanum

The addition of >100 μM La^{3+} (with excess DMN) irreversibly converted the usual light-evoked negative current response to a small positive current (Fig. 12). This effect could not be reversed by the addition of more caged Ca^{2+}. The positive La^{3+}-dependent current was also unaffected by the addition of DMN-Mg^{2+} or treatment with 5 μM monensin (*not shown*). These small, La^{3+}-dependent positive currents were also observed in proteoliposomes loaded with KCl buffer, and in experiments in which choline chloride substituted for the usual KCl in the external medium, but were not observed with liposomes without reconstituted protein. These results indicate that La^{3+} blocks the Na/Ca exchanger-mediated current.

Discussion

Functional Na/Ca Exchanger from Lobster Muscle can be Partially Purified and Incorporated into Proteoliposomes

The present report demonstrates that crustacean striated muscle possesses a Na/Ca exchanger similar to the exchanger present in mammalian heart. Western blots re-

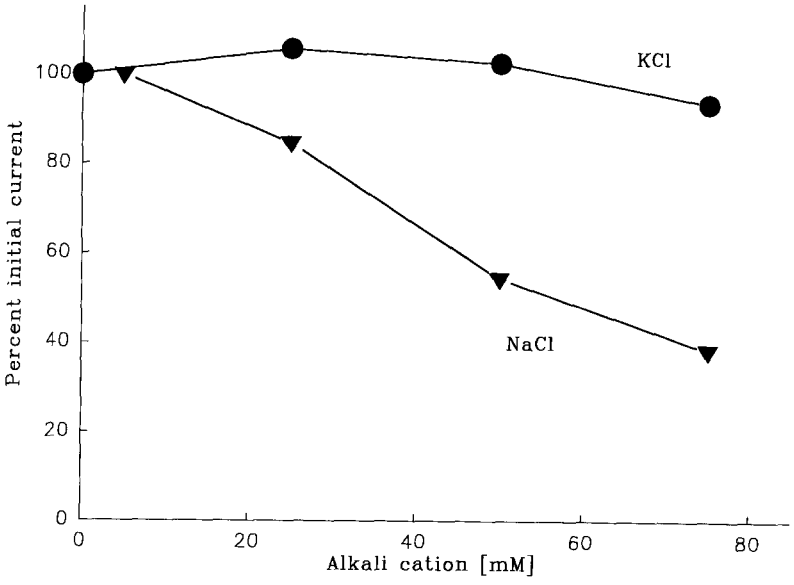

Fig. 9. Effects of NaCl and KCl on the light-evoked current. Starting medium = 180 mM Tris-HCl, pH 7.5 with (trans compartment, only) 600/200/200 μM DMN/Ca/Mg. The data are normalized to the stable peak amplitude immediately before the addition of the alkali cations (100%); in both cases, the trans compartment also contained 5 mM NaCl as a result of the addition of proteoliposomes (5.7 μg protein) suspended in NaCl buffer.

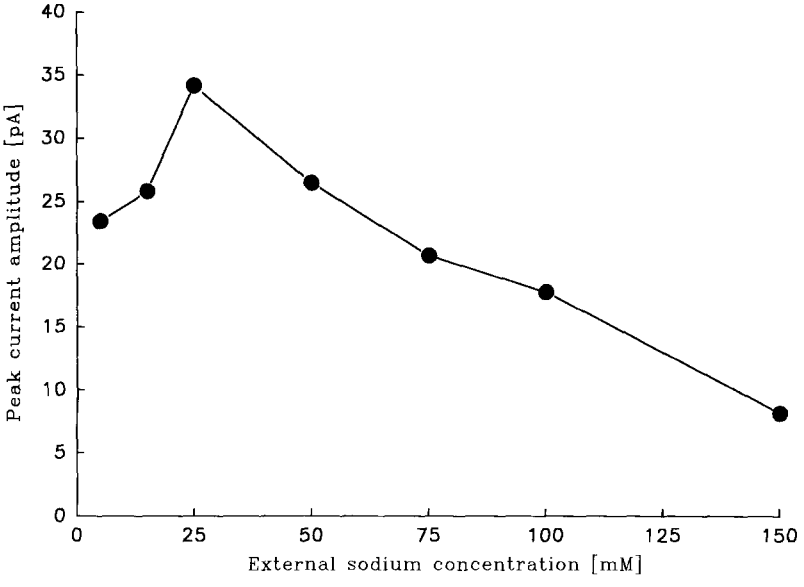

Fig. 10. Effect of Na^+ concentration on peak light-evoked current showing the activation and inhibition of the current by Na^+. Medium = KCl buffer. The trans compartment contained 300/200/200 μM DMN/Ca/Mg. The final Na^+ concentrations, following serial additions of NaCl buffer to this compartment, are indicated on the abscissa. The negative sign of the current has been ignored. The proteoliposome protein concentration was 3 μg.

veal that proteins in the lobster plasma membrane cross-react specifically with polyclonal antibodies raised against the canine cardiac Na/Ca exchanger. The specifically crossreacting bands on immunoblots as well as the major bands on silver stained gels of partially purified exchanger have molecular masses of 120 and 70 kDa. These bands are comparable in size to the exchanger bands detected in mammalian cardiac sarcolemma (Ambesi, Bagwell & Lindenmayer, 1991*a*, Durkin et al. 1991*a*, Nicoll et al. 1990, *see* Figs. 3 and 4). The smaller band (observed here as a doublet) appears to be a proteolytic fragment of the exchanger molecule (Nicoll et al., 1990; Durkin et al., 1991*a*). These data indicate that the structure of the cardiac-type Na/Ca exchanger molecule (*cf.* Nicoll et al., 1990; Nicoll & Philipson, 1991; Kofuji, Lederer & Schulze, 1994) is highly conserved among phylogenetically divergent species.

Proteoliposomes containing the partially purified lobster muscle Na/Ca exchanger exhibit a Na^+ gradient-dependent Ca^{2+} flux: Ca^{2+} uptake is promoted when $[Na^+]_i \geqslant [Na^+]_o$. This demonstrates that the partially purified and reconstituted exchanger is functional. The apparent similarity to the mammalian cardiac muscle Na/Ca exchanger indicates that the invertebrate muscle Na/Ca exchanger may be a very useful model preparation for biochemical and functional studies.

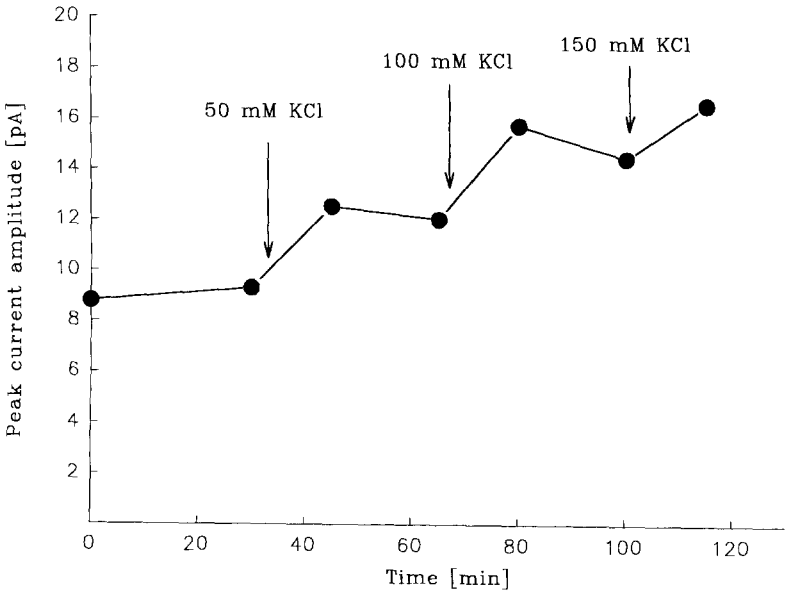

Fig. 11. Effect of K^+ on the light-evoked response in media containing 95 mM NaCl. At the start of the experiment, the medium contained (in mM): 160 N-methylglucamine, 95 NaCl, and 20 Tris-HCl pH 7.5. The trans compartment also contained 600/200/200 μM DMN/Ca/Mg and 3.2 μg proteoliposome protein. The indicated K^+ concentrations are final concentrations. The negative sign of the current has been ignored.

The Na/Ca Exchanger Cycle can be Detected in Proteoliposomes as a Ca^{2+} Concentration Jump-Evoked Current

The exchanger-containing proteoliposomes can be adsorbed to a positively charged BLM. When an outwardly-directed Na^+ gradient is present across the proteoliposome membranes, release of Ca^{2+} from a photolabile caged Ca^{2+} (DMN-Ca^{2+}) (Kaplan & Ellis-Davies 1988) into the extravesicular medium then induces a Ca^{2+} concentration jump and an outwardly-directed current under short circuit conditions. With the convention used here, the negative sign of the currents indicates that positive charges are moving out of the proteoliposomes. This is consistent with the outward movement of 3 Na^+ in exchange for one entering Ca^{2+}; it corresponds to the 3 Na^+:1 Ca^{2+} coupling ratio of the Na/Ca exchanger in invertebrate muscle (Rasgado-Flores et al., 1989).

Control experiments demonstrated that this activity is dependent upon the presence of intact, functional exchanger molecules and an outwardly-directed Na^+ concentration gradient. The current was reduced by hydrolyzing the protein with trypsin, by preventing the Ca^{2+} concentration jump, and by raising the external Ca^{2+} concentration above 30 mM (to inhibit Ca^{2+} binding). Low external Na^+ concentrations (<30 mM) activated the Ca^{2+} dependent current. The current was specifically dependent upon internal Na^+, and no current was observed in KCl-loaded proteoliposomes. These data clearly indicate that the internal Na^+ is required to make the system electrogenic. The data raise the possibility that the reconstituted exchanger can perform complete transport cycles.

To determine whether the observed current could be

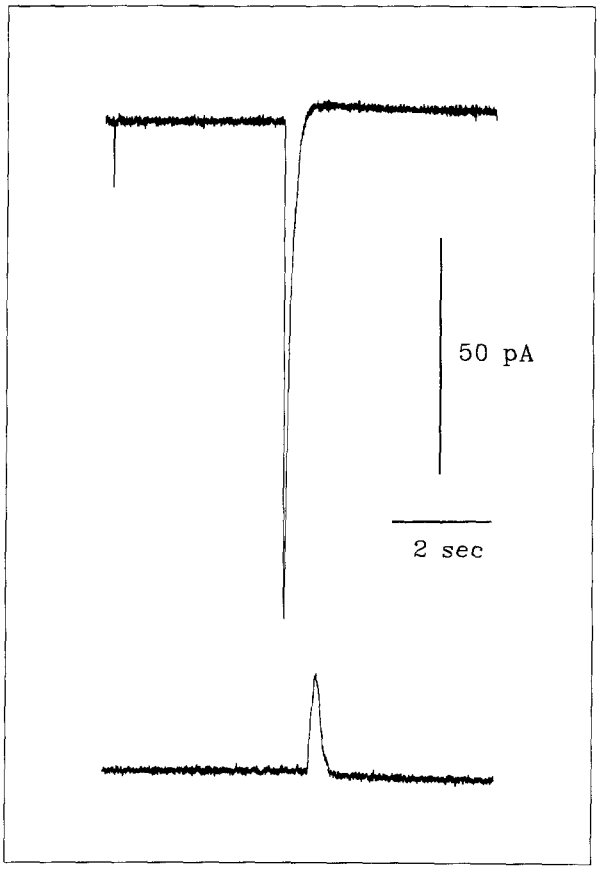

Fig. 12. Effect of La^{3+} on the light-evoked, Ca-dependent current. Medium = KCl buffer with (trans compartment only) 600/200 μM DMN/Ca and 5.7 μg proteoliposome protein. Upper record: the light-evoked current before addition of La^{3+}. Lower record: light-evoked current following the addition of DMN-La^{3+} (final concentrations: 600/200/200 μM DMN/Ca/La in the trans compartment).

attributed to cycling Na/Ca exchanger molecules, we estimated the amount of charge moved per exchanger. For simplicity, we began by assuming that the entire area of the hole between the two cuvette compartments (0.005 cm^2) was completely covered with 100 nm diameter liposomes, each containing one functional exchanger molecule. Accordingly, a maximum of 6.4×10^7 exchangers should be coupled to the BLM. Then, taking the largest light-evoked response that we observed (I_{max} = 341 pA), the integral of the peak area amounts to 2.15×10^{-11} coulombs. This means that 1.34×10^8 charges were moved out of the vesicles, or 2.1 charges per exchanger molecule. However, the number of coupled exchanger molecules was probably greatly overestimated, because: (i) At the high lipid to protein ratio used for reconstitution (*see above*), one exchanger molecule per vesicle may be the upper limit (Cheon & Reeves, 1988; Philipson et al., 1988), and (ii) It is likely that only a small fraction of the total BLM area is covered with vesicles (Bamberg et al., 1979). Moreover, the observed current response was probably somewhat attenuated by leakage current (Bamberg et al., 1979). Therefore, each exchanger molecule likely transported more than 2.1 net charges during each Ca^{2+} concentration jump. Taken together, the data are consistent with the possibility that the exchangers perform full cycles (with 3 Na^+ ions exiting the vesicles for each entering Ca^{2+}) in response to the Ca^{2+} concentration jumps.

We also must consider the amount of Na^+ that would need to be transferred to fit our results. Assuming that only about 2×10^5 100 nm diameter vesicles, each containing one exchanger molecules (= 2×10^5 exchangers), are coupled to the BLM, so that less than 1% of the BLM is covered with vesicles, the total vesicle volume is 10^{-13} liters. With NaCl buffer (160 mM NaCl) in the vesicles, the total number of Na^+ ions in the vesicles is about 10^{10}. If a maximum of 1.34×10^8 charges and, thus, 4×10^8 Na^+ ions (since the coupling ratio is 3 Na^+:1 Ca^{2+}; *cf.* Rasgado-Flores et al., 1989) leave the vesicles during a light flash (*see above*), this is about 4% of the total available intra-vesicular Na^+ ions. Of course, much smaller values are obtained if the smaller currents that we observed in most experiments are used for these calculations (e.g., about 0.3–1.0% of available Na^+).

The Properties of the Na/Ca Exchanger in the Proteoliposomes are Comparable to Those in Native Membranes

The aforementioned observations are consistent with the known properties of the Na/Ca exchanger: specific Na^+ gradient dependence, and activation by low Na^+ concentrations and inhibition by high Na^+ concentrations acting on the same side of the membrane as Ca^{2+}.

Activation of the currents by Ca^{2+} ions exhibited saturation kinetics with a $K_{M(Ca)} < 10$ μM (1 Ca^{2+} per binding site). This is consistent with $K_{M(Ca)}$ values for the intracellular Ca^{2+} binding site of the Na/Ca exchanger in squid axons (Blaustein, 1977; DiPolo & Beaugé, 1991) and mammalian cardiac muscle (Barcenas-Ruiz, Beuckelmann & Wier, 1987; Kimura & Miura, 1988; Hilgemann et al., 1991). The good fit of the Michaelis-Menten equation to our data (Fig. 6) suggests that only a single type of Ca^{2+} transport sites is involved in the generation of the observed currents. The apparent dissociation constant for the external Ca^{2+} binding site on the Na/Ca exchanger is in the low millimolar range in barnacle muscle (Rasgado-Flores & Blaustein, 1989), squid axons (Blaustein, Russell & De Weer, 1974; Blaustein, 1977) and mammalian muscle (Li et al., 1991). Thus, even though the proteoliposomes may contain mixtures of inside-out and right side-out exchanger molecules, only those with an inside-out orientation should have been activated under our experimental conditions ($[Ca^{2+}]_o \leq 40$ μM; *see* Fig. 6). The biphasic effect of sodium ions (activation of Na/Ca exchange at low Na^+ concentrations and inhibition at concentrations > 25 mM) has thusfar been observed only at the external surface of the exchanger (Baker et al., 1969; Blaustein, 1977; Reeves & Hale, 1984; Gadsby et al., 1991; Beaugé & DiPolo, 1991; Fontana, Rogowski & Blaustein, 1995). There are few studies on the effects of internal Na^+ (Blaustein & Russell, 1975; Beaugé & DiPolo, 1991), and in none have intracellular $[Na^+]$ in the range between 0 and 25 mM been tested. Therefore, our results (Fig. 10) raise the possibility that low concentrations of Na^+ at the intracellular binding site may also activate the Na/Ca exchanger.

In addition, we observed that photolysis of DMN evoked outward current when the Ca^{2+} was replaced by Sr^{2+} or Ba^{2+}, but not by Mg^{2+}. This is consistent with the known ability of the Na/Ca exchanger to mediate Na/Sr and Na/Ba (but not Na/Mg) exchange (Blaustein, 1977; Tibbits & Philipson, 1985). The Ca^{2+}-dependent current was blocked by La^{3+}. This fits with previous evidence that La^{3+} can block the Na/Ca exchanger (Baker et al., 1969; Rahamimoff & Spanier, 1984; Brommundt & Kavaler, 1987; Kimura et al., 1987; Caputo et al., 1989).

The observation of a light-evoked response in solutions containing caged Ni^{2+} (300 μM DMN:200 μM Ni^{2+}) was somewhat surprising in view of the known ability of Ni^{2+} to block Na/Ca exchanger-mediated currents in the heart (Kimura et al., 1987). However, much higher concentrations of Ni^{2+} (1–5 mM) are required for this block (Kimura et al., 1987) than were achievable in our experiments. It seems possible that Ni^{2+} may be transported by the exchanger (Brommundt & Kavaler, 1987) when the concentration is very low (perhaps about 20 μM), while much higher concentrations may block the exchanger. This possibility should be tested in tracer flux experiments.

In conclusion, the experiments described in this report demonstrate that functional Na/Ca exchangers from lobster muscle can be partially purified and incorporated into proteoliposomes. Moreover, when these proteoliposomes are adsorbed to a BLM, Ca^{2+} concentration jumps can be employed to activate the exchanger and induce Ca^{2+}-dependent electrogenic currents that can be attributed to the cycling of the exchanger. It should now be possible to study the individual steps in the exchanger cycle with appropriate modification of these methods.

The technical assistance of Verena Heiselpetz in some experiments is gratefully acknowledged. This work was partly supported by the Deutsche Forschungsgemeinschaft (SFB 169) and by National Institutes of Health grants HL30315 and GM39500 to JHK and HL45215 and NS16106 to MPB. MPB was the recipient of a Senior Scientist Award from the Alexander von Humboldt Stiftung.

References

Adrian, R.H., Chandler, W.K., Hodgkin, A.L. 1970. Voltage clamp experiments in striated muscle fibres. *J. Physiol.* **208:**607–644

Ambesi, A., Bagwell, E.E., Lindenmayer, G.E. 1991*a*. Purification and identification of the cardiac sarcolemmal Na/Ca exchanger. *Biophys. J.* **59:**138*a*

Ambesi, A., VanAlstyne, E.L., Bagwell, E.E., Lindenmayer, G.E. 1991*b*. Sequential use of detergents for solubilization and reconstitution of a membrane ion transporter. *Anal. Biochem.* **198:**312–317

Baker, P.F., Blaustein, M.P., Hodgkin, A.L., Steinhardt, R.A. 1969. The influence of calcium on sodium efflux in squid axons. *J. Physiol.* **200:**431–458

Bamberg, E., Apell, H.-J., Dencher, N.A., Sperling, W., Stieve, H., Läuger, P. 1979. Photocurrents generated by bacteriorhodopsin on planar bilayer membranes. *Biophys. Struct. Mech.* **5:**277–292

Barcenas-Ruiz, L., Beuckelmann, D.J., Wier, W.G. 1987. Sodium-calcium exchange in heart: membrane currents and changes in $[Ca^{2+}]_i$. *Science* **328:**1720–1722

Beaugé, L., DiPolo, R. 1991. Effects of monovalent cations on Na-Ca exchange in nerve cells. *Ann. N.Y. Acad. Sci.* **639:**147–155

Blaustein, M.P. 1977. Effects of internal and external cations and of ATP on sodium-calcium exchange in squid axons. *Biophys. J.* **20:**79–111

Blaustein, M.P. 1988. Calcium transport and buffering in neurons. *Trends Neuronsci.* **11:**438–443

Blaustein, M.P., Russell, J.M. 1975. Sodium-calcium exchange and calcium-calcium exchange in internally dialyzed squid axons. *J. Membrane Biol.* **22:**285–312

Blaustein, M.P., Russell, J.M., De Weer, P. 1974. Calcium efflux from internally dialyzed squid axons: The influence of external and internal cations. *J. Supramolec. Struct.* **2:**558–581

Blaustein, M.P., Goldman, W.F., Fontana, G., Krueger, B.K., Santiago, E.M., Steele, T.D., Weiss, D.N., Yarowsky, P.J. 1991. Physiological roles of the sodium-calcium exchanger in nerve and muscle. *Ann. N.Y. Acad. Sci.* **639:**254–274

Borlinghaus, R., Apell, H.J., Läuger, P. 1987. Fast charge translocation associated with partial reactions of the NaK pump. I. Current and voltage transients after photochemical release of ATP. *J. Membrane Biol.* **97:**169–178

Brommundt, G., Kavaler, F. 1987. La^{3+}, Mn^{2+}, and Ni^{2+} effects on Ca^{2+} pump and on Na^+-Ca^{2+} exchange in bullfrog ventricle. *Am. J. Physiol.* **253:**C45–C51

Caputo, C., Bezanilla, F., DiPolo, R. 1989. Currents related to the sodium-calcium exchange in squid axon. *Biochim. Biophys. Acta* **986:**250–256

Carafoli, E., Stauffer, T. 1994. The plasma membrane calcium pump: Functional domains, regulation of the activity, and tissue specificity of isoform expression. *J. Neurobiol.* **25:**312–324

Caroni, P., Reinlib, L., Carafoli, E. 1980. Charge movements during the Na^+-Ca^{2+} exchange in heart sarcolemmal vesicles. *Proc. Natl. Acad. Sci. USA* **77:**6354–6358

Cheon, J., Reeves, J.P. 1988. Site density of the Sodium-Calcium exchange carrier in reconstituted vesicles from bovine cardiac sarcolemma. *J. Biol. Chem.* **263:**2309–2315

Cook, N.J., Kaupp, U.B. 1988. Solubilization, purification and reconstitution of the sodium-calcium exchanger from bovine retinal rod outer segments. *J. Biol. Chem.* **263:**11382–11388

Costantin, L.L. 1977. Activation in striated muscle. *In:* Handbook of Physiology. The Nervous System Vol. I., pp. 215–259. American Physiological Society, Bethesda, MD

Dancshazy, Z., Karvaly, B. 1976. Incorporation of bacteriorhodopsin into bilayer lipid membrane: A photoelectric-spectroscopic study. *FEBS Lett.* **72:**136–138

Durkin, J.T., Ahrens, D.C., Pan, Y.-C.E., Reeves, J.P. 1991*a*. Purification and amino-terminal sequence of the bovine cardiac sodium-calcium exchanger: evidence for the presence of the a signal sequence. *Arch. Biochem. Biophys.* **290:**369–375

Durkin, J.T., Ahrens, D.C., Aceto, J.F., Condrescu, M., Reeves, J.P. 1991*b*. Molecular and functional studies of the cardiac sodium-calcium exchanger. *Ann. N.Y. Acad. Sci.* **639:**189–201

Eisenrauch, A., Bamberg, E. 1990. Voltage dependent pump currents of the sarcoplasmic reticulum Ca^{2+}-ATPase in planar bilayer membranes. *FEBS Lett.* **268:**152–156

Ellis-Davies, G.C.R., Kaplan, J.H. 1988. A new class of photolabile chelators for the rapid release of divalent cations: synthesis of caged Ca and caged *Mg. J. Org. Chem.* **153:**1966–1969

Fatt, P., Ginsborg, B.L. 1958. The ionic requirements for the production of action potentials in crustacean muscle fibres. *J. Physiol.* **142:**516–543

Fahr, A., Läuger, P., Bamberg, E. 1981. Photocurrent kinetics of purple-membrane sheets bound to planar bilayer membranes. *J. Membrane Biol.* **60:**51–62

Fendler, K., Grell, E., Haubs, M., Bamberg, E. 1985. Pump currents generated by the purified Na^+ K^+-ATPase from kidney on black lipid membranes. *EMBO J.* **4:**3079–3085

Fontana, G., Rogowski, R.S., Blaustein, M.P. 1995. Kinetic properties of the sodium/calcium exchanger in rat brain synaptosomes. *J. Physiol. (in press)*

Gadsby, D.C., Noda, M., Shepherd, R.N., Nakao, M. 1991. Influence of external nonovalent cations on Na-Ca exchange current-voltage relationships in cardiac myocytes. *Ann. N.Y. Acad. Sci.* **639:**140–146

Goldman, W.F., Yarowsky, P.J., Juhaszova, M., Krueger, B.K., Blaustein, M.P. 1994. Sodium/calcium exchange in rat cortical astrocytes. *J. Neurosci.* **14:**5834–5843

Hagiwara, S., Chichibu, S., Naka, K. 1964. The effects of various ions on resting and spike potentials of barnacle muscle fibers. *J. Gen. Physiol.* **48:**163–179

Hale, C.C., Slaughter, R.S., Ahrens, D.C., Reeves, J.P. 1984. Identification and partial purification of the cardiac sodium-calcium exchange protein. *Proc. Natl. Acad. Sci. USA* **81:**6569–6573

Hartung, K., Grell, E., Hasselbach, W., Bamberg, E. 1987. Electrical pump currents generated by the Ca-ATPase of sarcoplasmic reticulum vesicles adsorbed on black lipid membranes. *Biochim. Biophys. Acta* **900:**209–220

Hermann, T.R., Rayfield, G.W. 1976. A measurement of the proton current generated by bacteriorhodopsin in black lipid membranes. *Biochem. Biophys. Acta* **443:**623–628

Hilgemann, D.W., Collins, A., Cash, D.P., Nagel, G.A. 1991. Cardiac Na^+-Ca^{2+} exchange system in giant membrane patches. *Ann. N.Y. Acad. Sci.* **639:**126–139

Kaplan, J.H., Ellis-Davies, G.C.R. 1988. Photolabile chelators for the rapid photorelease of divalent cations. *Proc. Natl. Acad. Sci. USA* **85:**6571–6575

Kaplan, R.S., Pedersen, P.L. 1985. Determination of microgram quantities of protein in the presence of milligram levels of lipid with amido black 10B. *Anal. Biochem.* **150:**97–104

Kimura, J., Miura, Y. 1988. Effects of intracellular sodium and calcium on sodium-calcium exchange current. *J. Mol. Cell Cardiol.* **20:**S19

Kimura, J., Miyamae, S., Noma, A. 1987. Identification of sodium-calcium exchange current in single ventricular cells of guinea-pig. *J. Physiol.* **384:**199–222

Kofuji, P., Lederer, W.J., Schulze, D.H. 1994. Mutually exclusive and cassette exons underlie alternative spliced isoforms of the Na/Ca exchanger. *J. Biol. Chem.* **269:**5145–5149

Laemli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227:**680–685

Li, Z., Nicoll, D.A., Collins, A., Hilgemann, D.W., Filoteo, A., Penniston, J.T., Weiss, J.N., Tomich, J.M., Philipson, K.D. 1991. Identification of a peptide inhibitor of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *J. Biol. Chem.* **266:**1014–1020

Low, W., Kasir, J., Rahamimoff, H. 1993. Cloning of the rat heart Na^+-Ca^{2+} exchanger and its functional expression in HeLa cells. *FEBS Lett.* **316:**63–67

McCray, J.A., Fidler-Lim, N., Ellis-Davies, G.C.R., Kaplan, J.H. 1992. Rate of release of Ca^{2+} following laser photolysis of the DM-nitrophen-Ca^{2+} complex. *Biochemistry* **31:**8856–8861

Müller, P., Rudin, D.O., Tien, H.T., Wescott, W.C. 1962. Reconstitution of excitable cell membrane structure in vitro. *Circulation* **26:**1167–1171

Nicoll, D.A., Longoni, S., Philipson, K.D. 1990. Molecular cloning and functional expression of the cardiac sarcolemmal Na^+-Ca^{2+} exchanger. *Science* **250:**562–565

Nicoll, D.A., Philipson, K.D. 1991. Molecular studies of the cardiac sarcolemmal sodium-calcium exchanger. *Ann. N.Y. Acad. Sci.* **639:**181–188

Niggli, E., Lederer, W.J. 1991. Molecular operations of the sodium-calcium exchanger revealed by conformation currents. *Nature* **349:**621–624

Niggli, E., Lederer, W.J. 1993. Activation of Na-Ca exchange current by photolysis of "caged calcium". *Biophys. J.* **65:**882–891

Oakley, B.R., Kirsch, D.R., Morris, N.R. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105:**361–363

Philipson, K.D., McDonough, A.A., Frank, J.S., Ward, R. 1987. Enrichment of Na^+-Ca^{2+} exchange in cardiac sarcolemmal vesicles by alkaline extraction. *Biochim. Biophys. Acta* **899:**59–66

Philipson, K.D., Longoni, S., Ward, R. 1988. Purification of the Na^+-Ca^{2+} exchange protein. *Biochim. Biophys. Acta* **945:**298–306

Rahamimoff, H., Spanier, R. 1984. The asymmetric effect of lanthanides on Na^+-gradient-dependent Ca^{2+} transport in synaptic plasma membrane vesicles. *Biochem. Biophys. Acta* **773:**279–289

Rasgado-Flores, H., Santiago, E., Blaustein, M.P. 1989. Kinetics and stoichiometry of coupled Na efflux and Ca influx (Na/Ca exchange) in barnacle muscle cells. *J. Gen. Physiol.* **93:**1219–1241

Reeves, J.P., Hale, C.C. 1984. The stoichiometry of the cardiac sodium-calcium exchange system. *J. Biol. Chem.* **259:**7733–7739

Reeves, J.P., Sutko, J.L. 1983. Competitive interactions of sodium and calcium with the sodium-calcium exchange system of cardiac sarcolemmal vesicles. *J. Biol. Chem.* **258:**3178–3182

Riddell, F.G., Hayer, M.K. 1985. The monensin-mediated transport of sodium ions through phospholipid bilayers studied by ^{23}Na-NMR spectroscopy. *Biochim. Biophys. Acta* **817:**313–317

Ruscak, M., Orlicky, J., Juhaszova, M., Zachar, J. 1987*a*. Na^+-Ca^{2+} exchange in plasma membranes of crayfish striated muscle. *Gen. Physiol. Biophys.* **6:**469–478

Ruscak, M., Juhaszova, M., Orlicky, J., Zachar, J. 1987*b*. Reconstitution and partial purification of Na^+-Ca^{2+} exchange from crayfish striated muscle plasma membranes. *Gen. Physiol. Biophys.* **6:**523–528

Russell, J.M., Blaustein, M.P. 1974. Calcium efflux from barnacle muscle fibers: dependence on external cations. *J. Gen. Physiol.* **63:**144–167

Sheu, S.-S., Blaustein, M.P. 1992. Sodium/Calcium exchange and control of cell calcium and contractility in cardiac and vascular smooth muscle. *In:* The Heart and Cardiovascular System. 2nd Edition. H.A. Fozzard, E.A. Haber, R.B. Jennings, A.M. Katz, H.E. Morgan, editors. pp. 903–943. Raven, New York.

Tibbits, G.F., Philipson, K.D. 1985. Na^+-dependent alkaline earth metal uptake in cardiac sarcolemmal vesicles. *Biochim. Biophys. Acta* **817:**327–332

Towbin, H., Gordon, J. 1984. Immunoblotting and dot immunobinding—current status and outlook. *J. Immunol. Methods* **72:**313–340

van der Hijden, H.T.W.M., Grell, E., de Pont, J.J.H.H.M., Bamberg, E. 1990. Demonstration of the electrogenicity of proton translocation during the phosphorylation step in gastric H,K-ATPase. *J. Membrane Biol.* **114:**245–256