Purification and Identification of the Cardiac Sarcolemmal Na+Ca2+ Exchanger

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PURIFICATION AND IDENTIFICATION OF THE CARDIAC

SARCOLEMMAL Na+/Ca2+ EXCHANGER

By

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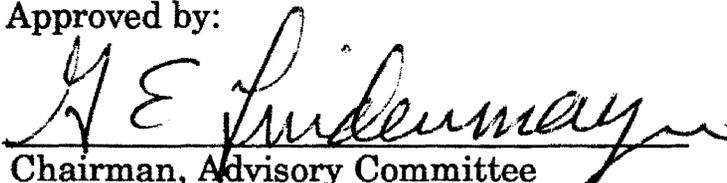
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ANTHONY AMBESI. Purification and Identification of the Cardiac Sarcolemmal Na+/Ca²⁺ Exchanger. (Under the direction of GEORGE E. LINDENMAYER, M.D., Ph.D. and ERVIN E. BAGWELL, Ph.D.)
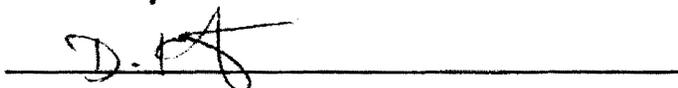
The cardiac sarcolemmal Na+/Ca²⁺ exchanger promotes the coupled movement of sodium and calcium across the cell membrane in a reversible, electrogenic manner. The goal of this study was to identify the protein(s) responsible for Na+/Ca²⁺ exchange across cardiac sarcolemma.

Purification of the Na+/Ca²⁺ exchanger from canine ventricle was accomplished using the following sequence: 1) isolation of sarcolemma (SL); 2) alkaline extraction of membranes; 3) solubilization with CHAPS; 4) DEAE chromatography; 5) gel filtration HPLC; 6) addition of cholate, salt and phospholipids; 7) wheat germ agglutinin chromatography followed by 8) reconstitution into proteoliposomes by detergent dilution. Specific activity was increased from 5.2 (SL) to 3766 nmol/mg/sec with 10% recovery of activity and 0.02% recovery of protein. SDS-PAGE of the purified Na+/Ca²⁺ exchanger preparation under reducing conditions revealed prominent proteins of 75, 120 and 140 kDa. One major protein with a molecular mass of 140 kDa was detected under nonreducing conditions. Polyclonal antibodies against the reconstituted, purified Na+/Ca²⁺ exchanger preparation recognized the three proteins and immunoprecipitated 97.4%±1.3% (n=4) of the Na+/Ca²⁺ exchange activity from detergent-solubilized sarcolemma. Subsequently, antibodies against the 75, 120

and 140 kDa proteins were antigen-purified and found to immunoprecipitate 92%, 91% and 83%, respectively, of the Na^+/Ca^{2+} exchange activity from detergent-solubilized sarcolemma. Furthermore, the antigen-purified antibodies cross-reacted with each of the other two proteins on immunoblots of sarcolemmal protein. Immunoblots with samples prepared from isolated canine ventricular myocytes revealed predominantly the 140 kDa protein and trace amounts of the 75 kDa protein. The 120 kDa protein was not detected.

The polyclonal antibodies were subsequently tested for the ability to affect Na^+/Ca^{2+} exchange activity manifested by sarcolemmal vesicles. IgG from immune serum stimulated exchange activity 3.5-fold in a concentration-dependent manner (ED_{50} = 0.5 μg IgG/μg sarcolemmal protein) while IgG from preimmune serum had little or no effect. Curiously, IgG from a rabbit immunized against total sarcolemmal protein stimulated activity nearly as much, but was unable to immunoprecipitate exchange activity. Stimulation appeared to be due to a small increase in V_{max} and a larger decrease in the $K_{0.5}$ for extravesicular calcium.

Molecular cloning of the canine cardiac Na^+/Ca^{2+} exchanger has recently been reported (Nicoll *et al.*, 1990). RNA synthesized from the cDNA clone induced expression of Na^+/Ca^{2+} exchange activity when injected into *Xenopus* oocytes. The cDNA codes for a protein of 970 amino acids (~108 kDa). Antibodies developed against a synthetic peptide based on the deduced amino acid sequence reacted with

sarcolemmal proteins of 70, 120 and 160 kDa. Antibodies generated against a partially purified canine cardiac Na^+/Ca^{2+} exchanger preparation also recognized proteins of 70, 120 and 160 kDa (Philipson *et al.*, 1988). These antibodies (provided by K. D. Philipson) recognized proteins of 75, 120 and 140 kDa on immunoblots of sarcolemmal protein and the purified Na^+/Ca^{2+} exchanger preparation in this laboratory (Ambesi *et al.*, 1991c).

These data support the hypothesis that the 70-75, 120 and 140-160 kDa proteins are involved with Na^+/Ca^{2+} exchange across cardiac sarcolemma and that the 70-75 and 120 kDa proteins are fragments of the 140-160 kDa protein formed during isolation of the sarcolemmal preparation.

ACKNOWLEDGEMENTS

DEDICATION

To my parents, Anthony and Shirley Ambesi; wife, Johneen; and our daughter Nicole. With love and gratitude.

CHAPTER I

General Introduction

The existence of a plasmalemmal Na^+/Ca^{2+} exchange system was first hypothesized just over 20 years ago when Reuter and Seitz (1968) demonstrated that calcium efflux in atria was sensitive to extracellular concentrations of free sodium and calcium and that both cations interacted with a common site associated with the plasma membrane. Later, Baker *et al.* (1969) provided more direct evidence for the existence of a plasma membrane Na^+/Ca^{2+} exchanger using internally perfused squid giant axons and further proposed that a Na^+/Ca^{2+} exchange system could explain the positive inotropic effect observed with cardiac glycosides. Research on the cardiac Na^+/Ca^{2+} exchanger has received much attention as a result of the known regulatory effects of Na^+ and Ca^{2+} on cardiac excitation-contraction coupling, implicating Na^+/Ca^{2+} exchange as a key modulator of myocardial contractility.

In light of the recent advances in both experimental design and techniques, the role of Na^+/Ca^{2+} exchange in cardiac excitation-contraction coupling is slowly beginning to emerge with emphasis being placed upon myocardial relaxation (Ca^{2+} efflux) as its primary function. However, the Na^+/Ca^{2+} exchanger can also operate in a Ca^{2+} influx mode suggesting a role in Ca^{2+}-mediated cardiac contraction. Thus, the physiological role of the Na^+/Ca^{2+} exchanger in the regulation of intracellular Ca^{2+} in cardiac tissue remains somewhat controversial. Uncertainty concerning the physiological role can be attributed to a

number of factors including: (1) the difficulty in obtaining purified preparations of the cardiac Na^+/Ca^{2+} exchanger; (2) the lack of selective inhibitors of Na^+/Ca^{2+} exchange; and (3) the presence of multiple Na^+ and Ca^{2+} flux pathways which greatly increases the complexity of cardiac excitation-contraction coupling (Figure 1).

While the original goal of this study was to identify the protein(s) responsible for Na^+/Ca^{2+} exchange across cardiac sarcolemma (SL) and not necessarily to purify them, the results presented herein, and those reported in the literature subsequent to the initiation of this project, suggest that we have developed fractionation procedures which purify the cardiac Na^+/Ca^{2+} exchanger to near-homogeneity. Purification of the protein(s) responsible for Na^+/Ca^{2+} exchange and manipulation of cDNA clones encoding the active exchanger should ultimately permit a closer inspection of its biochemical properties and provide additional insight into its physiological role in cardiac excitation-contraction coupling.

In this introductory chapter, several aspects of Na^+/Ca^{2+} exchange will be presented and discussed including: (1) stoichiometry and thermodynamics, (2) kinetics, (3) regulation, (4) physiological role of Na^+/Ca^{2+} exchange and (5) discussion of past and present attempts to purify the proteins responsible for Na^+/Ca^{2+} exchange across cardiac sarcolemma.
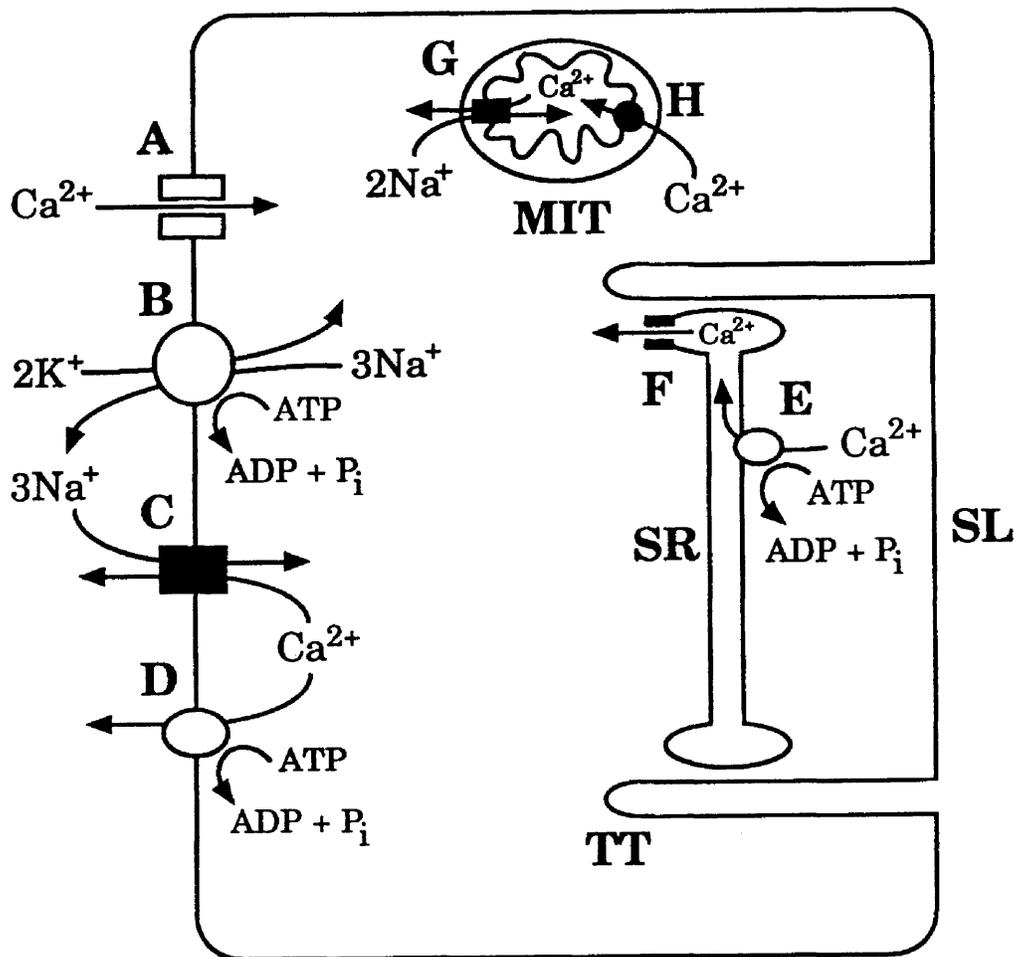
Figure 1.   **Ion Transport Pathways in a Cardiac Myocyte which Play a Role in the Regulation of Intracellular Ca $^{2+}$.** Ca $^{2+}$ is transported either into or out of the cytosol in response to physiological signals (e.g., changes in ion gradients, depolarization, ligand binding). A, L-type Ca $^{2+}$-channel (voltage-gated); B, Na $^+$,K $^+$-ATPase; C, Na $^+$/Ca $^{2+}$ exchanger; D, sarcolemmal Ca $^{2+}$-ATPase (calmodulin-sensitive); E, sarcoplasmic reticulum Ca $^{2+}$-ATPase (calmodulin-insensitive); F, sarcoplasmic reticulum Ca $^{2+}$ release channels (Ca $^{2+}$-dependent and receptor-operated); G, mitochondrial Na $^+$/Ca $^{2+}$ exchanger; H, mitochondrial Ca $^{2+}$ uniporter; MIT, mitochondrion; SL, sarcolemma; SR, sarcoplasmic reticulum; TT, transverse tubule.

STOICHIOMETRY AND THERMODYNAMICS OF Na+/Ca²⁺ EXCHANGE

The stoichiometry of Na+/Ca²⁺ exchange dictates the potential physiological significance of Na+/Ca²⁺ exchange in regulating intracellular free calcium concentration. For example, a stoichiometry of 2 Na+:1 Ca²⁺ implies an electroneutral exchange process which is independent of membrane potential and would be unable to reduce intracellular calcium to basal levels (Blaustein, 1974). Moreover, evidence supporting electrogenic exchange suggests that the stoichiometry is greater than 2 Na+:1 Ca²⁺. Studies designed to determine the stoichiometry of Na+/Ca²⁺ exchange were carried out initially in intact squid giant axons and gave results consistent with 2-5 Na+ transported per Ca²⁺ (Baker *et al.*, 1969; Blaustein and Russel, 1975). Intracellular organelles, however, can accumulate or release calcium and lead to incorrect estimates.

Subsequently, plasma membrane vesicles were used to measure isotopic fluxes of Na+ and Ca²⁺. Pitts (1979) used simultaneous initial rate measurements of ²²Na+ influx and ⁴⁵Ca²⁺ efflux in cardiac sarcolemmal vesicles and demonstrated a stoichiometry of 3 Na+:1 Ca²⁺. Wakabayashi and Goshima (1981a) reported similar results by measuring calcium flux in fetal mouse cardiac cells. Reeves and Hale (1984) generated potential differences across cardiac sarcolemmal vesicles to drive Na+/Ca²⁺ exchange while determining the Na+ gradient necessary to prevent net Ca²⁺ flux. Their results further supported a

stoichiometry of 3 Na^+:1 Ca^{2+}. Through measurement of Na^+/Ca^{2+} exchange currents in isolated guinea-pig ventricular cells, Kimura *et al.* (1987) determined a reversal potential consistent with a stoichiometry of 3 Na^+:1 Ca^{2+}. In addition to those cited above, there have been numerous reports supporting a stoichiometry of 3 Na^+:1 Ca^{2+} (Bers *et al.*, 1980; Caroni *et al.*, 1980; Philipson and Nishimoto, 1980; Reeves and Sutko, 1980; Reeves *et al.*, 1980; Sheu and Fozzard, 1982; Allen *et al.*, 1983; Kimura *et al.*, 1987).

Besides squid axon, a stoichiometry of 3 Na^+:1 Ca^{2+} has also been reported for Na^+/Ca^{2+} exchange in certain noncardiac tissues including vascular smooth muscle (Ashida and Blaustein, 1987; Kahn *et al.*, 1988) and barnacle muscle (Rasgado-Flores and Blaustein, 1987). In contrast, the Na^+/Ca^{2+} exchanger localized to the outer segment plasma membrane of retinal rod cells exhibits a requirement for K^+ and has a stoichiometry of 4 Na^+:1 Ca^{2+} + 1 K^+ (Lagnado and McNaughton, 1989). Recent evidence, however, suggests that the cardiac sarcolemmal Na^+/Ca^{2+} exchanger protein is structurally different (separate gene products with no homology) from that of retinal rod cells (Cook *et al.*, 1991).

The relationship between the concentration of intracellular free calcium and the other determinants of the exchange reaction at equilibrium can be expressed by the equation shown below based on a stoichiometry of 3 Na^+:1 Ca^{2+} (Blaustein, 1974):

$$[Ca^{2+}]_i = [Ca^{2+}]_o \left(\frac{[Na^+]_i}{[Na^+]_o}\right)^3 \exp\left(\frac{E_mF}{RT}\right)$$

Where i and o refer to free cation concentrations in the intracellular and extracellular spaces, respectively; E_m is the membrane potential; F is the Faraday constant; R is the gas constant; and T is the absolute temperature in degrees Kelvin.

The above equation predicts $[Ca^{2+}]_i$ for Na^+/Ca^{2+} exchange at equilibrium. Given that the cell exists in a steady state under physiological conditions, the usefulness of the equation is limited to predicting the *direction* of net calcium flux via Na^+/Ca^{2+} exchange. For example, if the $[Ca^{2+}]_i$ calculated from the equation were higher than the actual concentration, then the exchanger would tend to move net calcium into the cell in an attempt to reach equilibrium for the exchange reaction. Substituting appropriate values for $[Ca^{2+}]_o$, $[Na^+]_i$ and $[Na^+]_o$ (1.5, 7.5 and 144 mM, respectively; Philipson, 1990) at 37° and a membrane potential of -85 mV (phase 4 of the action potential), the $[Ca^{2+}]_i$ calculated from the equation is ~ 0.009 µM (one order of magnitude lower than the 0.06-0.3 µM typically observed; Sheu *et al.*, 1984; Chapman, 1986). Thus, the equation predicts that the exchanger will move calcium in the direction of net calcium efflux in an attempt to reach equilibrium. Subsequent to the onset of an action potential and membrane depolarization (approximately 20 mV; phase 0 of the action potential), $[Ca^{2+}]_i$ initially remains near basal levels. Under equilibrium conditions for the

exchange reaction, the equation predicts an $[Ca^{2+}]_i$ (0.5 μM) which is approximately 2-8-fold greater than resting calcium (0.06-0.3 μM). Thus, Na^+/Ca^{2+} exchange favors net calcium influx. Yet, depolarization activates voltage-dependent Ca^{2+}-channels in the plasma membrane which leads to Ca^{2+}-induced Ca^{2+} release from internal stores and an increase in $[Ca^{2+}]_i$ to ~ 0.6-10 μM (phase 2 of the action potential). Under these conditions, the equation predicts that the exchanger will begin to reverse direction and move calcium out of the cell. Furthermore, one might predict that the driving force for calcium efflux via Na^+/Ca^{2+} exchange would be greatest immediately following membrane repolarization (phase 3 of the action potential), given that $[Ca^{2+}]_i$ is elevated and the cell is repolarized.

From a thermodynamic standpoint, it appears that Na^+/Ca^{2+} exchange in cardiac muscle alternates between Ca^{2+}-efflux and Ca^{2+}-influx modes depending upon the state of the cell. The relative contribution of each transport mode to calcium homeostasis, however, remains uncertain and will be addressed below under "Physiological Role".

KINETICS

The physiological role of Na^+/Ca^{2+} exchange in cardiac excitation-contraction coupling will depend upon how effectively the exchanger can compete

with other calcium transport pathways in the cardiac cell. This in turn will depend upon various kinetic parameters of Na^+/Ca^{2+} exchange including the exchanger's affinity (K_M) for internal and external Na^+ and Ca^{2+} and how quickly (V_{max}) it can transport Ca^{2+} across the cell membrane. A great deal of data concerning the kinetics of Na^+/Ca^{2+} exchange have been acquired from studies using cellular or tissue preparations and, in particular, sarcolemmal vesicles.

Because of the technical difficulties associated with patch clamp studies on whole cell preparations, such as the presence of multiple cellular compartments and the need to block other Ca^{2+} transport pathways, determination of kinetic parameters is accompanied by much uncertainty. However, cellular preparations offer the advantage of being in a more physiological state. Conversely, membrane preparations offer a system in which quantitative data can easily be obtained; and in fact, much of the data reported in the literature has been obtained from isolated membrane preparations. However, kinetic data generated from vesicular preparations are not free of uncertainty. For example, isolation of membrane preparations may result in the loss of endogenous regulators or alteration of the proteins responsible for exchange activity. Furthermore, membrane preparations are typically comprised of both inside-out and right-side-out oriented vesicles.

Nonetheless, the cardiac Na^+/Ca^{2+} exchanger appears to be a relatively high capacity, high turnover transporter with a relatively low affinity for Ca^{2+} (Philipson, 1990).

Ca²⁺ Transport Site

Typical values for the $K_{0.5}$ for Ca²⁺ based on Na⁺$_i$-dependent Ca²⁺ uptake into cardiac sarcolemmal vesicles range from 10 to 40 μM (e.g., Table 1 in Reeves and Philipson, 1989). The use of Ca²⁺ chelators such as EGTA has been reported to lower the $K_{0.5}$ for Ca²⁺ to ~1 μM (Caroni *et al.*, 1980; Caroni and Carafoli, 1983; Trosper and Philipson, 1984). Assessment of the exchanger's affinity for Ca²⁺ in vesicular preparations, however, may be erroneous due to the presence of vesicles with mixed polarities. One might intuitively expect the exchanger to exhibit high affinity for Ca²⁺ on the intracellular surface and low affinity for Ca²⁺ on the extracellular surface. If this were so, and Na⁺/Ca²⁺ exchange were asymmetric, then ion flux measurements across sarcolemmal vesicles would display two compartment kinetics and curvature would be observed in Eadie-Hofstee plots. This has not been reported. Philipson and Nishimoto (1982a) took advantage of the presence of the Na⁺,K⁺-ATPase to load inside-out cardiac sarcolemmal vesicles with Na⁺. Na⁺$_i$-dependent Ca²⁺ uptake was then measured and compared with that measured in the total vesicular population. The affinity for calcium on the intracellular surface (33 μM) was reported to be identical to that on the extracellular surface (see also Philipson, 1985).

In more intact cellular preparations using isolated cardiac myocytes, the $K_{0.5}$ reported for extracellular Ca²⁺ is typically much higher ranging from 150 μM to 1.4 mM suggesting that there is indeed some asymmetry with respect to the

exchanger's affinity for Ca^{2+} on the intracellular and extracellular surfaces (Wakabayashi and Goshima, 1981b; Kimura *et al.*, 1987; Vemuri *et al.*, 1989). Studies of exchange activity in internally perfused squid axons support this notion in that the apparent $K_{0.5}$ for Ca^{2+} is different for external and internal Ca^{2+} transport sites (3 mM and 10 µM, respectively; Baker and McNaughton, 1978).

Na+ Transport Site

The data concerning Na+ binding to the Na+/Ca^{2+} exchanger appears less controversial with respect to binding asymmetry (Philipson, 1985). Na+-dependent Ca^{2+} transport exhibits a sigmoidal dependency on Na+ concentration with a Hill coefficient of 2-3 which is consistent with the proposed 3 Na+ : 1 Ca^{2+} stoichiometry (Philipson and Nishimoto, 1981; Wakabayashi and Goshima, 1981a; Kadoma *et al.*, 1982; Reeves and Sutko, 1983; Caroni and Carafoli, 1983; Philipson, 1985; de la Pena and Reeves, 1987). The $K_{0.5}$ for Na+ typically falls within the range of 20 to 30 mM and has been reported from studies using both cardiac sarcolemmal vesicles and cardiac cells (Wakabayashi and Goshima, 1981a; Kimura and Miura, 1988).

The observation that Na+ and Ca^{2+} compete for ion binding sites on the exchanger (competitive inhibition; K_i for Na+ is ~16 mM with a Hill coefficient of 2 at high [Na+]) has led to the development of a model in which two binding sites (site A and site B) on each side of the membrane were proposed (Reeves and Sutko, 1983). At the A-site (Ca^{2+} binding site), either one Na+ or two Na+ can bind where

only one Na+ is necessary to effectively prevent Ca²⁺ binding. Binding of two Na+ to the A-site results in a conformational change which allows a third Na+ to bind to the B-site. According to this model, occupation of both the A-site and B-site by three Na+ and the A-site by Ca²⁺ on the trans membrane side is required for Na+/Ca²⁺ exchange.

Alternate Transport Modes

Interestingly, the Na+/Ca²⁺ exchanger has also been shown to undergo Ca²⁺-Ca²⁺ exchange (Bartschat and Lindenmayer, 1980; Philipson and Nishimoto, 1981; Slaughter *et al.*, 1983; Philipson *et al.*, 1988) and Na+-Na+ exchange (DiPolo and Beauge, 1987). In addition, alkali metal ions have been shown to stimulate Ca²⁺-Ca²⁺ exchange (K~Rb~Li>Cs>>choline) by increasing the V_{max} (Coutinho *et al.*, 1983; Philipson and Nishimoto, 1981; Slaughter *et al.*, 1983).

Capacity and Turnover Rate

As previously mentioned, the cardiac Na+/Ca²⁺ exchanger is a relatively high capacity Ca²⁺ transporter. V_{max} rates of 5-30 nmol Ca²⁺ transported/mg/sec are consistently reported for cardiac sarcolemmal vesicles which translates to Ca²⁺ flux rates of ~10-30 pmol/cm²/sec in the intact cardiac cell (Chapman, 1983). Flux rates reported in membrane vesicles prepared from brain (Schellenberg and Swanson, 1981; Gill *et al.*, 1984), pituitary (Kaczorowski *et al.*, 1984), and vascular

1 3

smooth muscle (Morel and Godfraind, 1984), are typically much lower. More recent results using isolated synaptosomal preparations from rat brain, however, suggest that the brain Na^+/Ca^{2+} exchanger may be as active as that of cardiac tissue (Fontana and Blaustein, 1991).

The turnover rate has been estimated to be about 1000 Ca^{2+} sec^{-1} for the cardiac exchanger reconstituted into proteoliposome preparations by Cheon and Reeves (1988) using indirect methods. Niggli and Lederer (1991) estimated the turnover rate to have an upper limit of about 2500 Ca^{2+} sec^{-1} based on Na^+/Ca^{2+} exchange currents in isolated guinea-pig myocytes using patch clamp techniques. Estimates of the turnover rate based on purified cardiac Na^+/Ca^{2+} exchanger preparations range from ~150 Ca^{2+} sec^{-1} (Philipson et al., 1988) to 527 Ca^{2+} sec^{-1} at V_{max} (see chapter III). Conversely, the turnover rate estimated from activities of purified rod outer segment Na^+/Ca^{2+} exchange preparations is somewhat lower at 30-50 Ca^{2+} sec^{-1} (Cook and Kaupp, 1988; Nicoll and Applebury, 1989).

REGULATION

The modulation of Na^+/Ca^{2+} exchange activity in cardiac and noncardiac membrane preparations and in intact cells by a number of factors has been reported. The physiological significance of each of these factors has not yet been established. The mechanisms thought to modulate Na^+/Ca^{2+} exchange activity

are:

(1)   Phosphorylation of the exchanger;

(2)   regulation by intracellular Ca^{2+};

(3)   modulation by pH;

(4)   activation by proteolysis;

(5)   modulation by constituents of the membrane environment.

Phosphorylation of the Exchanger

Stimulation of Na^+/Ca^{2+} exchange activity with ATP was initially reported in internally dialyzed squid axons (Blaustein, 1977; DiPolo and Beauge, 1983; DiPolo, 1985; DiPolo and Beauge,1986; DiPolo and Beauge, 1987; DiPolo and Beauge, 1988). Activation of exchange activity exhibited a requirement for internal Mg^{2+} (DiPolo and Beauge, 1984) and was found to be selective for ATP (ADP, GTP, UTP, ITP, AMP, cAMP, acetylphosphate, and phosphoarginine were ineffective; DiPolo, 1976). While hydrolyzable ATP analogues (α,β-methylene ATP and 2-deoxy-ATP) also stimulated exchange activity, the non-hydrolyzable ATP analogue β,γ-methylene ATP was ineffective (DiPolo, 1977). Subsequently, DiPolo and Beauge (1987) demonstrated activation of the exchanger with [γ-S]ATP which is a substrate for protein kinases but not for ATPases. These results are consistent with the interpretation that the ATP effect is the result of phosphorylation mediated by a

calcium-dependent protein kinase. The mechanism by which ATP stimulates exchange activity in squid axon appears to be by increasing the exchanger's affinity for Ca^{2+} on the intracellular surface at the Ca^{2+} regulatory site (discussed below) from ~10-15 μM to ~1-4 μM (DiPolo and Beauge, 1988).

The regulation of Na^+/Ca^{2+} exchange activity by ATP has also been reported in barnacle muscle (Blaustein, 1977; DiPolo and Beauge, 1983) and cultured aortic smooth muscle cells (Vigne *et al.*, 1988). Evidence for ATP stimulation of Na^+/Ca^{2+} exchange in cardiac tissue is less certain. Carafoli and associates reported activation of exchange activity with ATP in sarcolemmal vesicles using a Ca^{2+}-calmodulin-dependent protein kinase and phosphatase (Caroni and Carafoli, 1983; Reinlab *et al.*, 1987). However, others have been unable to reproduce their results (Philipson, 1990).

Hilgemann (1991) used giant excised membrane patches from cardiac myocytes to demonstrate a stimulatory effect of Mg^{2+}-ATP (K_M (ATP) ~1.5 mM) on exchange activity. While the effect could be blocked with pentalysine (binds to phosphatidylserine head groups), protein kinase and phosphatase inhibitors had no effect. Hilgemann (1991) postulated that the mechanism by which ATP exerts its modulatory effect in cardiac cells was indirect, via an aminophospholipid translocase which establishes membrane asymmetry (phosphatidylserine and anionic membrane components stimulate exchange activity, discussed below).

Regulation by Intracellular Ca^{2+}

An interesting finding is the absolute requirement of the exchanger for internal Ca^{2+} in order to mediate Na^+_i-dependent Ca^{2+} influx. Although first reported in squid axon (Baker and McNaughton, 1976; DiPolo, 1979; DiPolo and Beauge, 1986; DiPolo and Beauge, 1987), internal Ca^{2+} regulatory sites have also been reported in cardiac cells (Kimura *et al.*, 1986; Kimura and Miura, 1988; Noda *et al.*, 1988), cardiac sarcolemmal preparations (Reeves and Poronnik, 1987) and barnacle muscle (Rasgado-Flores and Blaustein, 1987). ATP has been reported to increase the affinity for Ca^{2+} at the Ca^{2+} regulatory site ($K_{0.5}$ decreased from 10-15 μM to 1-4 μM; DiPolo and Beauge, 1988). Calcium binding at the regulatory site has been shown to increase the affinity for Na^+ and the maximal rate of exchange (DiPolo and Beauge, 1988). In internally perfused ventricular myocytes, the $K_{0.5}$ for Ca^{2+} at the regulatory site has been reported to be ~20-50 nM (Kimura and Miura, 1988; Noda *et al.*, 1988) which may suggest differences between the cardiac and squid axon exchangers.

Reeves and Poronnik (1987) looked at the modulation of Na^+/Ca^{2+} exchange activity in bovine cardiac sarcolemmal vesicles by intravesicular Ca^{2+}. While Na^+/Ca^{2+} exchange activity in sarcolemmal vesicles exhibited no absolute requirement for intravesicular Ca^{2+}, internal Ca^{2+} was reported to increase the affinity of the exchanger for extravesicular Ca^{2+}. However, the effect could not be saturated even at values of 1 mM intravesicular Ca^{2+} and was, therefore,

suggested to be nonspecific having no bearing on the Ca^{2+} regulatory site (Reeves and Poronnik, 1987; de la Pena and Reeves, 1987). A hypothesis proposed by Hilgemann (1988) suggests that the Ca^{2+} regulatory site may serve as a protective mechanism for the cell by preventing the exchanger from lowering intracellular Ca^{2+} too low. Thus, as intracellular Ca^{2+} levels drop below ~0.1 μM, Ca^{2+} would dissociate from the Ca^{2+} regulatory site and Ca^{2+} efflux via the exchanger would cease.

Modulation by pH

Na^+/Ca^{2+} exchange activity in heart displays sigmoidal dependency on pH increasing from pH 6 to pH 9 (Wakabayashi and Goshima, 1981b; Philipson *et al.*, 1982; Slaughter *et al.*, 1983). The effect of pH on Na^+/Ca^{2+} exchange activity has also been reported in squid axon (DiPolo and Beauge, 1982). At low pH, protons compete with Ca^{2+} for Ca^{2+} binding sites on the exchanger, thereby inhibiting Na^+/Ca^{2+} exchange activity. This may have significance in certain pathological conditions in which intracellular pH is altered (e.g. pH_i is lowered in ischemic myocardium).

Activation by Proteolysis

While activation of Na^+/Ca^{2+} exchange activity in cardiac sarcolemmal vesicles by limited protease treatment has been reported (Philipson and

Nishimoto, 1982), assigning a physiological relevance to proteolysis in the *in vivo* situation is more difficult. One might conceive that under certain physiological states or pathological conditions, selective proteolysis of the Na^+/Ca^{2+} exchanger could occur and increase Na^+/Ca^{2+} exchange activity. Whether activation of the exchanger occurs through proteolysis of the exchanger itself, inhibitory proteins or both is unclear. Nonetheless, proteolysis of the cardiac Na^+/Ca^{2+} exchanger clearly occurs during membrane isolation and purification of the exchanger (Philipson *et al.*, 1988; see chapter III) and has delayed conclusive identification of the proteins responsible for Na^+/Ca^{2+} exchange across cardiac sarcolemma.

Modulation by Constituents of the Membrane Environment

The effects of the lipid environment in cardiac sarcolemmal and reconstituted preparations on Na^+/Ca^{2+} exchange activity has been extensively studied. In general, exchange activity is enhanced in the presence of certain anionic membrane constituents and diminished in the presence of certain cationic membrane constituents. Treatment of sarcolemmal vesicles with phospholipase C (preferentially hydrolyzes neutral phospholipids resulting in a membrane relatively enriched with negatively charged phospholipids; Philipson *et al.*, 1983) and phospholipase D (converts neutral phospholipids to phosphatidic acid; Philipson and Nishimoto, 1984) were reported to enhance Na^+/Ca^{2+} exchange activity 2- and 4-fold, respectively.

In reconstitution experiments where the membrane components are easily manipulated, Vemuri and Philipson (1988) showed that cardiolipin and phosphatidylserine were required for high exchange rates. Small anionic amphiphiles such as sodium dodecylsulfate (SDS) and fatty acids added to sarcolemmal vesicles were also shown to enhance Na^+/Ca^{2+} exchange activity (Philipson, 1984; Philipson and Ward, 1985; Philipson and Ward, 1987; Vemuri and Philipson, 1989).

PHYSIOLOGICAL ROLE

The physiological role of Na^+/Ca^{2+} exchange in cardiac excitation-contraction coupling has been a subject of much debate and controversy. In light of the recent advances in patch-clamp techniques, the development of Ca^{2+} sensitive dyes and pharmacological and biochemical approaches, the role of Na^+/Ca^{2+} exchange as a major Ca^{2+} efflux system involved in myocardial relaxation is beginning to unfold.

Following the onset of an action potential, Ca^{2+} enters the cytosol from both extracellular (through L-type Ca^{2+} channels) and intracellular stores (through Ca^{2+} release channels in the sarcoplasmic reticulum). Subsequently, an equivalent amount of Ca^{2+} must be removed from the cytosol prior to the beginning

of the next excitation-contraction cycle in order to maintain Ca^{2+} homeostasis. Ca^{2+} is sequestered into sarcoplasmic reticular stores via the SR ATP-dependent Ca^{2+} pump. In contrast, there are two systems in the plasma membrane of the cardiac cell which return calcium to the extracellular space: (1) the SL ATP-dependent Ca^{2+} pump and (2) the Na^+/Ca^{2+} exchanger. The question as to the relative contribution of each of these systems to Ca^{2+} removal and cardiac relaxation has been addressed in a number of studies (Murphy *et al.*, 1986; Barry *et al.*, 1986; Bridge *et al.*, 1988; Bers and Bridge, 1989; Bridge *et al.*, 1990; Cannel, 1991; Bers, 1991).

Caffeine added to a myocyte or muscle superfusate (inhibits SR Ca^{2+}-ATPase) decreased the rate of relaxation by ~70% while a Na^+-free and Ca^{2+}-free superfusate (inhibits Na^+_o-dependent Ca^{2+} efflux) slowed relaxation by ~30% (Bers, 1991). Furthermore, relaxation was almost completely abolished when Na^+/Ca^{2+} exchange and the SR Ca^{2+}-ATPase were simultaneously blocked. Cannel (1991) obtained similar results from patch-clamp studies using voltage-clamped, single cardiac myocytes while measuring intracellular Ca^{2+}. In these studies, Na^+/Ca^{2+} exchange was reported to contribute little to the rise in intracellular Ca^{2+} leading to contraction, but was responsible for ~15% of the Ca^{2+} efflux during relaxation. Bridge *et al.*, (1990) treated guinea pig ventricular myocytes with caffeine in the absence of extracellular sodium and induced contraction by depolarization (voltage clamp from -40 mV to 0 mV). Upon repolarization, however, the cells were unable

to relax due to inhibition of the SR Ca^{2+}-ATPase and the Na^+/Ca^{2+} exchanger. Addition of extracellular Na^+ caused immediate relaxation and was attributed to Na^+_o-dependent calcium efflux via the Na^+/Ca^{2+} exchanger. These data suggest that Na^+/Ca^{2+} exchange accounts for ~80-100% of the Ca^{2+} efflux from cardiac cells and ~15-30% of the total Ca^{2+} removed from the cytosol. Moreover, the contribution of the SL Ca^{2+}-ATPase to Ca^{2+} efflux and cardiac relaxation appears to be negligible.

Evidence that Na^+/Ca^{2+} exchange may contribute to Ca^{2+} influx subsequent to the onset of an action potential (see "Stoichiometry and Thermodynamics of Na^+/Ca^{2+} exchange") leads one to suspect that Na^+/Ca^{2+} exchange may also play a role in cardiac contraction. Leblanc and Hume (1990) treated guinea pig ventricular myocytes with 5 μM nisoldipine (L-Type Ca^{2+}-channel blocker) and demonstrated an elevation in $[Ca^{2+}]_i$ upon membrane depolarization which was dependent on sodium influx through tetrodotoxin-sensitive Na^+-channels. In addition, the rise in intracellular calcium was dependent on $[Ca^{2+}]_o$. These data suggest that calcium entry via Na^+/Ca^{2+} exchange may contribute to Ca^{2+}-induced Ca^{2+} release from intracellular stores and myocardial contraction. Lederer *et al.* (1990) point out that the rise in $[Na^+]_i$ during membrane depolarization would be inadequate to elevate $[Ca^{2+}]_i$ sufficiently via Na^+/Ca^{2+} exchange to induce calcium release from the SR. Restricting Na^+ diffusion to just below the membrane surface, however, may result in sufficiently high $[Na^+]_i$ in the vicinity of the

exchanger to allow enough Ca^{2+} to enter the cell through Na^+/Ca^{2+} exchange and, thereby, to induce Ca^{2+} release from the SR (Lederer *et al*, 1990).

It seems clear that one role of Na^+/Ca^{2+} exchange in cardiac relaxation is to remove from the cytosol an amount of Ca^{2+} equal to that which enters through voltage-dependent Ca^{2+} channels in the plasma membrane during the plateau phase of the action potential. The role of Na^+/Ca^{2+} exchange in Ca^{2+}-induced Ca^{2+} release remains less certain.

PURIFICATION

In recent years, there have been a number of attempts to identify and isolate the proteins responsible for Na^+/Ca^{2+} exchange across cardiac sarcolemma (Hale *et al.*, 1984; Soldati *et al.*, 1985; Philipson *et al.*, 1988; Durkin *et al.*, 1990; Ambesi *et al.*, 1990; Durkin *et al.*, 1991; Ambesi *et al.* , 1991). Attempts at identification were based on a correlation between a specific protein band upon SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and increased transport activity. While meaningful increases in specific activity were obtained (see below), none of the earlier attempts to purify the exchanger were conclusive in identifying the protein(s) responsible for Na^+/Ca^{2+} exchange. Some of the difficulty faced in identifying and isolating the Na^+/Ca^{2+} exchanger can be attributed to three considerations: (1) The Na^+/Ca^{2+} exchanger is a high-turnover, low abundance

protein (Philipson *et al.*, 1988; Ambesi *et al.*, 1990; Ambesi *et al.* 1991a; see chapter III; Durkin *et al.*, 1991); (2) there are no specific, high affinity ligands which can be used to follow the exchanger during fractionation or coupled to affinity supports for purification (Kaczorowski *et al.*, 1989); and (3) subsequent to fractionation, all samples must be reconstituted into sealed proteoliposomes for assay of exchange activity (i.e., based on Na$^+$-dependent Ca^{2+} flux). To further complicate matters, limited proteolysis (Philipson and Nishimoto, 1982b), alkaline pH (Wakabayashi and Goshima, 1981b; Philipson *et al.*, 1982; Slaughter *et al.*, 1983; Vemuri and Philipson, 1988), phospholipase treatment (Philipson *et al.*, 1983; Philipson and Nishimoto, 1984) and incorporation of certain components into reconstituted vesicles (Philipson, 1984; Philipson and Ward, 1985; Philipson and Ward, 1987; Vemuri and Philipson, 1988; Vemuri and Philipson, 1989) can have dramatic effects on activity (see "Regulation" above).

In order to assess purification of exchange activity following fractionation, one must take into account the loss of exchanger units during fractionation and the incorporation of exchanger units from leaky vesicles in the sarcolemmal preparation (exchange activity not detected) into sealed proteoliposomes (exchange activity detected). Thus, purification is typically calculated relative to activities of control proteoliposomes (solubilized and reconstituted sarcolemmal protein) instead of the sarcolemma-enriched preparation (starting source for purification). However, several considerations should be noted: (1) the sarcolemmal preparation

used in this laboratory as the starting source for purification of the Na^+/Ca^{2+} exchanger is substantially enriched with sarcolemmal markers (i.e., approximately 66-fold over homogenate; VanAlstyne *et al.*, in preparation); and (2) I am convinced that some purification occurs by solubilization and reconstitution per se, although, the amount cannot be specified because the extent of loss of exchanger units and degree of activation are currently unknown (see chapter II).

Hale *et al.* (1984) solubilized bovine cardiac sarcolemmal vesicles with cholate in the presence of soybean phospholipids (asolectin), treated the supernatant with Pronase, and increased specific activity 30-fold in reconstituted proteoliposomes when compared to native sarcolemmal vesicles (native sarcolemmal vesicles = 4.1 nmols/mg/sec; control reconstituted vesicles = 19.7 nmols/mg/sec; Pronase treated, reconstituted vesicles = 128 nmol/mg/sec). Enrichment correlated with an 82 kDa band on SDS-PAGE. However, later reports suggested that the 82 kDa protein could be separated from Na^+/Ca^{2+} exchange activity (Philipson *et al.*, 1988).

Soldati *et al.* (1985) solubilized the Na^+/Ca^{2+} exchanger from a bovine cardiac sarcolemma-enriched preparation with Triton X-100 in the presence of asolectin, fractionated activity by rate zonal centrifugation, and enriched Na^+/Ca^{2+} exchange activity 128-fold over native sarcolemma (sarcolemmal preparation = 7.1 nmols/mg/sec; reconstituted vesicles = 94.4 nmols/mg/sec; fractionated activity =

908 nmols/mg/sec). Activity appeared to correlate with a 33 kDa band on SDS-PAGE. However, a large number of protein bands could be detected in their most enriched preparation.

Prior to solubilization with cholate/asolectin and reconstitution by detergent dilution, Philipson et al. (1987) treated canine cardiac sarcolemmal vesicles with an alkaline medium (10 mM cyclohexylaminopropane sulfonic acid (CAPS)-NaOH, pH 12; suggested to remove loosely bound peripheral proteins; Steck and Yu, 1983). Specific activity in reconstituted, alkaline-extracted sarcolemmal vesicles was enriched 2-fold over that of control reconstituted vesicles with little or no loss of exchange activity (reconstituted control vesicles = 31 nmols/mg/sec; reconstituted alkaline-extracted vesicles = 64 nmols/mg/sec). Using alkaline extraction, DEAE-Sepharose chromatography, and immobilized wheat germ agglutinin chromatography in series, Philipson et al. (1988) were able to enrich Na^+/Ca^{2+} exchange activity 27-fold over control reconstituted vesicles (sarcolemma = 5 nmols/mg/sec; reconstituted vesicles = 24 nmols/mg/sec; fractionated activity = 654 nmols/mg/sec). Activity appeared to correlate with 70 and 120 kDa bands on SDS-PAGE under reducing conditions and 160 kDa under nonreducing conditions. Curiously, prolonged exposure to chymotrypsin resulted in the disappearance of the 70 and 120 kDa bands with little or no loss of activity.

Polyclonal antibodies directed against the enriched fraction immunoprecipitated 96% of the Na^+/Ca^{2+} exchange activity from detergent-

solubilized sarcolemma (Philipson *et al.*, 1988). Later, Vemuri *et al.* (1990) developed monoclonal antibodies against their partially purified exchanger preparation. Antibodies from three independent clones were shown to immunoprecipitate 50-75% of the exchanger. Additional studies confirmed that proteins precipitated by the monoclonal antibodies were the same as those recognized by the polyclonal antibodies previously developed (Philipson *et al.*, 1988). The polyclonal antibodies generated against the partially purified exchanger preparation (Philipson *et al.*, 1988) were used to screen an amplified λgt11 canine heart cDNA expression library and a partial clone (lacked the 5' coding region) was isolated (Nicoll *et al.*, 1990). DNA probes developed from the clone were subsequently used to identify a cDNA clone from a canine heart oligo(dT)-primed cDNA library which had a 5' extension of 80 base pairs. RNA synthesized from this clone and injected into *Xenopus laevis* oocytes induced expression of Na^+_i-dependent Ca^{2+} uptake and provided strong evidence that the clone coded for the cardiac Na^+/Ca^{2+} exchanger (Nicoll *et al.*, 1990).

OBJECTIVE AND SPECIFIC AIMS

Objective

The objective of this project was to identify the protein(s) responsible for

Na$^+$/Ca^{2+} exchange across cardiac sarcolemma.

Specific Aims

The specific aims of the project were as follows:

(1) To develop an improved solubilization and reconstitution procedure using zwitterionic and non-ionic detergents.

(2) To develop a means to enrich the protein(s) responsible for Na$^+$/Ca^{2+} exchange through ion-exchange chromatography.

(3) To develop a means to enrich the protein(s) responsible for Na$^+$/Ca^{2+} exchange through gel filtration chromatography.

(4) To enrich further the protein(s) responsible for Na$^+$/Ca^{2+} exchange by combining ion-exchange and gel filtration chromatography in series.

(5) To test alternative hypotheses about the protein(s) responsible for Na$^+$/Ca^{2+} exchange.

Chapter II addresses Specific Aim 1, solubilization and reconstitution of the cardiac Na$^+$/Ca^{2+} exchanger, and has been accepted by *Analytical Biochemistry* for publication (Ambesi *et al.*, 1991e).

Chapters III and IV address Specific Aims 2-5, purification and identification of the cardiac sarcolemmal Na$^+$/Ca^{2+} exchanger, and have been submitted to the *Journal of Biological Chemistry* for publication (Ambesi *et al.*, 1991b and Ambesi *et al.*, 1991c, respectively). In addition, abstracts presented at

meetings of the *Biophysical Society* also cover Specific Aims 2-5 (Ambesi *et al.*, 1990; Ambesi *et al.*, 1991a).

Chapter V includes data characterizing the effects of polyclonal antibodies on Na^+/Ca^{2+} exchange activity. These results were presented at the *New York Academy of Sciences* "Second International Conference on Sodium/Calcium Exchange" and are in press for the proceedings of the conference (Ambesi *et al.*, 1991d).

Chapter VI is a general discussion of the data presented here and in the literature concerning the identification of the protein(s) responsible for Na^+/Ca^{2+} exchange across cardiac sarcolemma.

CHAPTER II

Sequential Use of Detergents for Solubilization and Reconstitution of a Membrane Ion Transporter

INTRODUCTION

The purification of integral membrane proteins almost invariably requires solubilization of the protein with detergent followed by the application of one or more column chromatography procedures (Renswoude and Kempf, 1984). After purification, the protein must often be reconstituted into a lipid bilayer environment to assess function. One means to rapidly effect reconstitution in the presence of certain detergents is to dilute the mixed micellar system to reduce the level of the detergent to the point that proteoliposomes are formed (Racker *et al.*, 1979; Koepsell, 1986). In this regard, the anionic detergent cholate has been successfully employed for the solubilization and reconstitution of a number of integral membrane proteins (Racker *et al.*, 1979; Racker, 1979; Koepsell, 1986). However, the use of cholate during purification has disadvantages (e.g., precludes ion exchange chromatography, see Renswoude and Kempf, 1984; Hjelmeland and Chrambach, 1984; may interfere with certain lectin affinity separations, see Lotan *et al.*, 1977; Renswoude and Kempf, 1984). Thus, purification may be benefitted by solubilization with another detergent, fractionation in the presence of that detergent, followed by the addition of cholate and lipids for the reconstitution step.

Miyamoto and Racker (1980) first reported solubilization and reconstitution of the Na^+/Ca^{2+} exchanger by use of cholate in the presence of soybean

phospholipids and high ionic strength. This paper reports modifications of the Miyamoto and Racker procedure (1980). The modifications allow an improved recovery of total and specific Na^+/Ca^{2+} exchange activity upon reconstitution. In addition, the combination of solubilization with other detergents followed by reconstitution via the cholate dilution technique was examined. This sequence was found to be useful for several detergent combinations and could have utility for other integral membrane proteins.

MATERIALS AND METHODS

Materials

3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), n-octyl-ß-D-glucoside, and Triton X-100 were obtained from Pierce Chemical Co.; decyl-ß-D-maltoside was from Calbiochem; cholic acid was from Serva; and ^{45}Ca was from New England Nuclear. Asolectin (L-α-phosphatidylcholine, Type II-S) was obtained from Sigma Chemical Co., and was treated as previously described (Hale *et al.*, 1984). All other chemicals were of reagent grade.

Sarcolemma Enriched Preparations

Cardiac ventricular tissue was removed from anesthetized dogs and

minced as previously described (VanAlstyne *et al.*, 1980; Frankis and Lindenmayer, 1984). The following steps were carried out at 3-5°C, pH values are for 5°C, and centrifugal forces are given as max RCF (relative centrifugal force). The mince was suspended in 5 volumes of 15 mM $NaHCO_3$, 0.25 M sucrose, pH 7.0 and subjected to one pass of a motor-driven Teflon pestle in a Potter-Elvehjem vessel (clearance approx. 0.318 mm). After centrifugation (1600 x g; 10 min), the pellet was rehomogenized and pelleted as described above. The pellet was then resuspended in 5 volumes of 10 mM Tris-Cl, pH 7.2 by four motor-driven passes of a pestle (clearance approx. 0.254 mm). After centrifugation as above, the supernatant was collected. This step was repeated three times. The four supernatants were centrifuged (39,000 x g; 20 min), the pellets were separately resuspended in 0.25 volumes of 10 mM Tris-Cl, pH 7.0, and each layered over 15 ml of 0.771 M sucrose, 10 mM Tris-Cl, pH 7.4 in Beckman Type 30 rotor tubes. After centrifugation (73,000 x g; 30 min), the layer at the interface between the buffer and sucrose was harvested, diluted with 15-20 ml 10 mM Tris-Cl, pH 7.4, and centrifuged (73,000 x g; 20 min). The pellets were washed once in the same buffer, resuspended at 2-4 mg/ml, frozen in liquid nitrogen, and stored at -70°C.

Solubilization and Reconstitution of the Na^+/Ca^{2+} Exchanger

Membrane vesicles (1.5 mg/ml) were exposed to 160 mM NaCl, 10 mM 4-morpholinepropanesulfonic acid (Mops)-Tris, pH 7.4 (Buffer I), for 12-18 hours on

ice (Na+-loading). Solubilization with cholate was carried out by a twofold dilution of the vesicle suspension with a solution containing cholate, asolectin, NaCl (concentrations specified under "Results and Discussion"), 10 mM Mops-Tris, pH 7.4. Insoluble material was removed by centrifugation (180,000 x g; 30 min). Proteoliposomes were formed by dilution with Buffer I, collected by centrifugation (230,000 x g; 60 min), and washed once by resuspension and centrifugation. Proteoliposomes were resuspended in 100-300 µl of Buffer I and assayed for Na+/Ca^{2+} exchange activity and protein.

Solubilization with detergents other than cholate was carried out by a twofold dilution of the Na+-loaded sarcolemmal vesicles with the detergent dissolved in Buffer I. Exposure to detergent was carried out on ice for 30 min with frequent vortexing. Subsequently, insoluble material was removed by centrifugation (180,000 x g; 30 min). Reconstitution by cholate dilution was achieved by diluting the solubilized fraction with an equal volume of 2% cholate, 800 mM NaCl, 10 mM Mops-Tris, pH 7.4, 50 mg/ml asolectin which had been sonicated to a uniform suspension with a bath sonifier (Model W185F, Heat Systems-Ultrasonics, Inc.) and cleared of insoluble material by centrifugation (180,000 x g; 30 min). After incubation on ice for 5 min, proteoliposomes were formed by a threefold dilution with Buffer I, collected by centrifugation, and washed once as described above.

Assay for Na+/Ca²⁺ Exchanger

Na+/Ca²⁺ exchange assays were carried out at 37°C for one sec according to the procedure of Reeves and Sutko (1983) using 40 μM ⁴⁵Ca/CaCl₂ and 0.5 μM valinomycin and a stopping solution of 160 mM KCl, 1 mM EGTA, 10 mM Mops-Tris, pH 7.4.

Miscellaneous

Protein was determined by the method of Lowry *et al.* (1951) for sarcolemmal preparations. Protein of proteoliposome preparations was determined by modifications of the Schaffner and Weissmann procedure (1973) as described by Kaplan and Pedersen (1985). Bovine serum albumin (BSA) was used as a standard for all protein assays. The amount of insoluble material in the cholate/phospholipid suspension (prior to removal by centrifugation) was estimated by 90° light scatter at 550 nm using an Aminco Bowman spectrophotofluorometer.

RESULTS AND DISCUSSION

Miyamoto and Racker (1980) reported solubilization of the Na+/Ca²⁺ exchanger by exposing sarcolemmal membranes (2 mg/ml) to 2% cholate, 500 mM

NaCl, 24 mg/ml asolectin, 20 mM 4-morpholineethanesulfonic acid (Mes)-Tris, pH 7.4 for 20 min on ice. Following removal of insoluble material, reconstitution was achieved by a sixfold dilution of the solubilized preparation with 160 mM NaCl, 20 mM Mes-Tris, pH 7.4. This procedure resulted in a fivefold increase in specific activity with no change in total activity. Since that time, there have been a number of reports on the use of cholate to solubilize Na^+/Ca^{2+} exchange activity from cardiac tissue (Hale *et al.*, 1984; Luciani, 1984; Carpenedo *et al.*, 1986; Vemuri and Philipson, 1987; Hale *et al.*, 1988). Specific activity was typically increased four- to fivefold over that of native sarcolemmal vesicles.

Here we report solubilization and reconstitution conditions which increase specific activity more than 30-fold over that of native sarcolemmal vesicles. Optimal recovery of Na^+/Ca^{2+} exchange activities was found to occur at a solubilizing concentration of 1% cholate (Figure 2). The difference between this result and that of Miyamoto and Racker (1980) may reflect the lower protein concentration used here for solubilization (0.75 vs. 2.0 mg/ml). Similar values were obtained with and without the 20-min incubation used by Miyamoto and Racker (1980). Recovery of activity was found to be dependent on the extent of dilution with a threefold dilution resulting in optimal recovery of total activity (Figure 3). This result is equivalent to that of Miyamoto and Racker (1980; used a sixfold dilution) given that the cholate concentration after dilution was identical (0.33%). Presumably, the degree of dilution affects the probability for conversion of
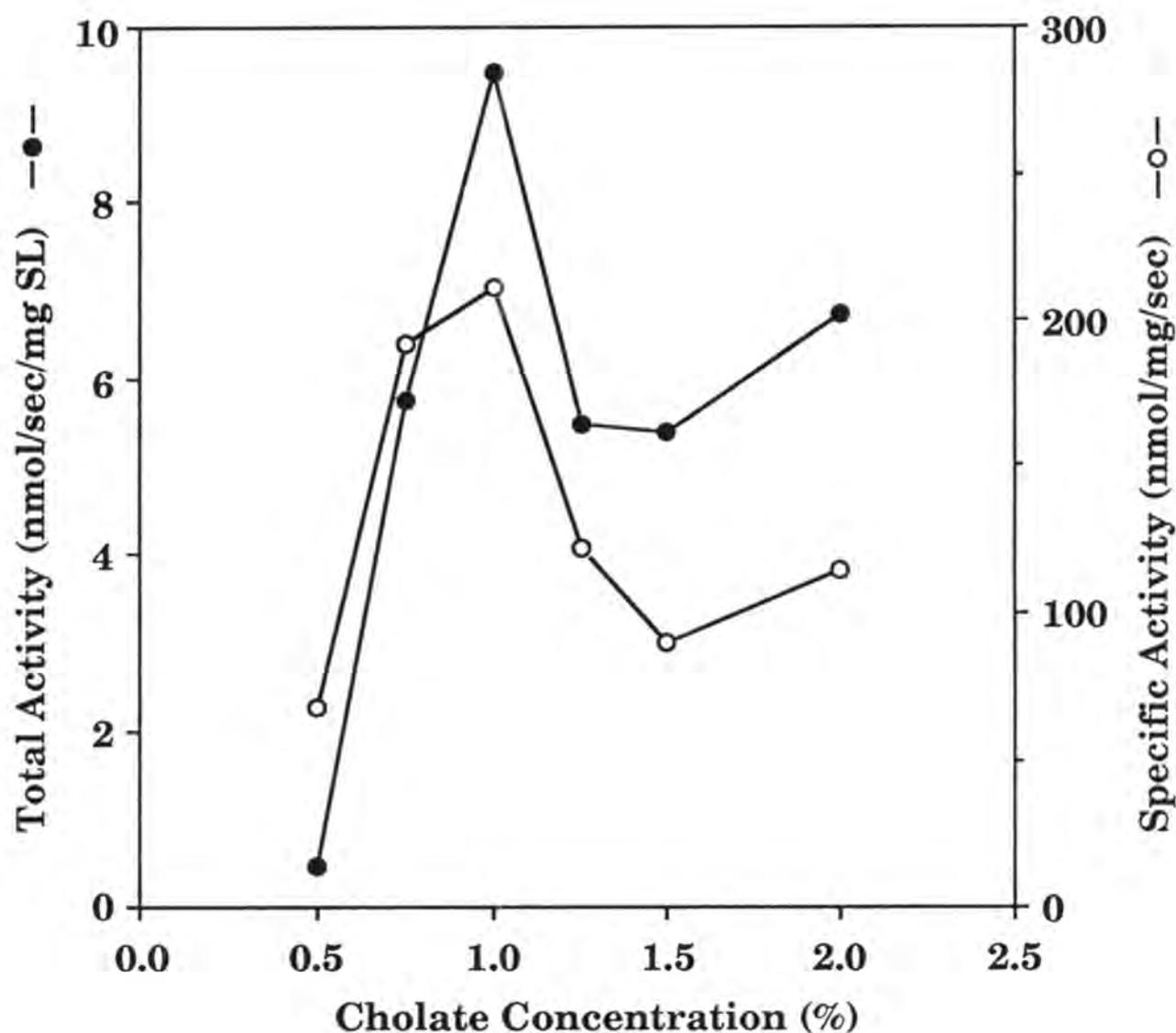
Figure 2. **Recovery of Na+/Ca2+ Exchange Activity Versus Cholate Concentration.**
Na+-loaded sarcolemmal vesicles were solubilized with cholate as
specified in 480 mM NaCl, 10 mM Mops-Tris, pH 7.4, 25 mg/ml
asolectin. Insoluble material was removed by centrifugation and
protein was reconstituted by a sixfold dilution with Buffer I and assayed
for Na+/Ca2+ exchange activity as described under "Materials and
Methods" (n = 2).
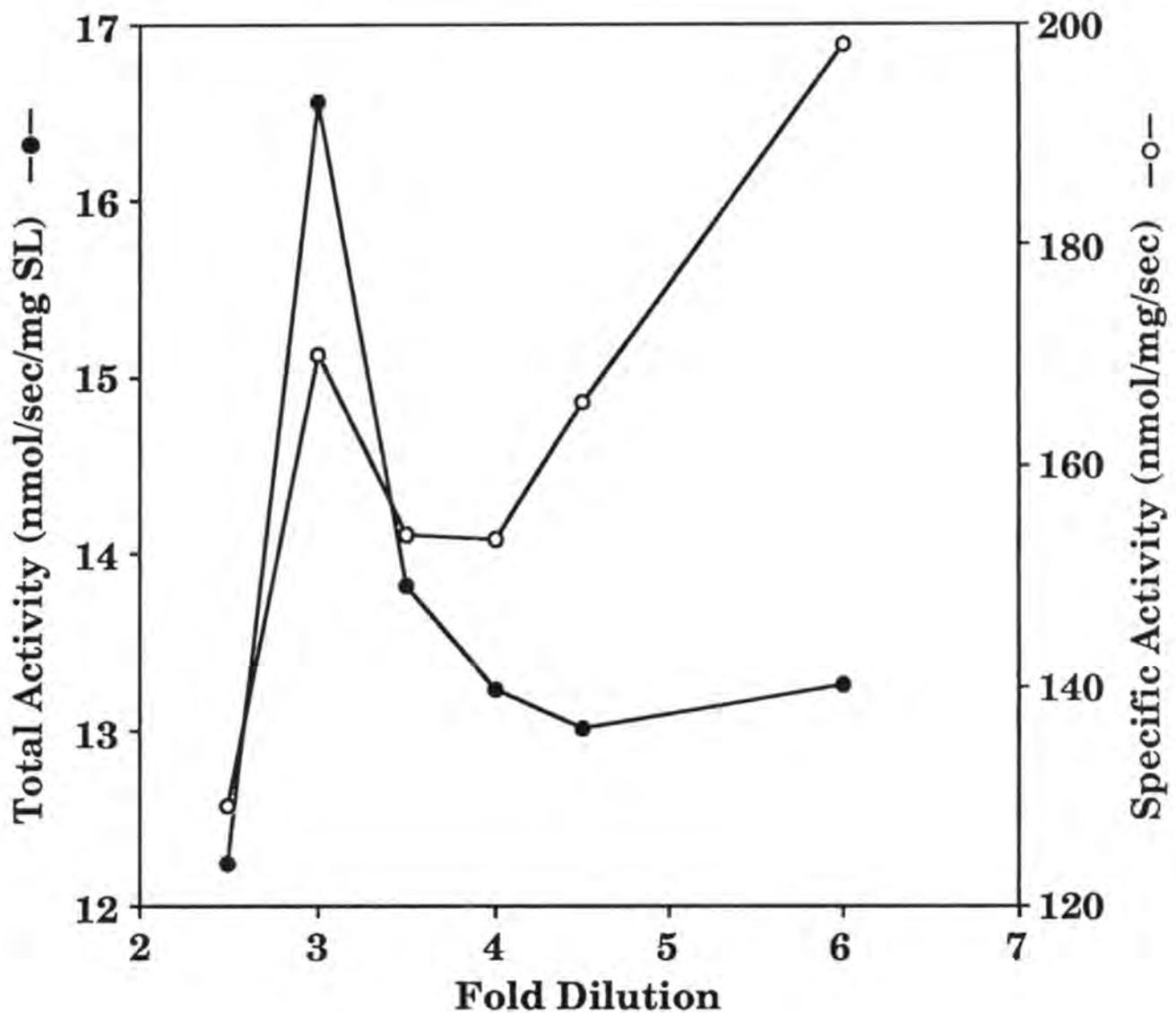
Figure 3. **Recovery of Na$^+$/Ca^{2+} Exchange Activity Versus Dilution of Cholate-solubilized Fraction for Reconstitution.** Na$^+$-loaded sarcolemmal vesicles were solubilized with 1% cholate, 480 mM NaCl, 10 mM Mops-Tris, pH 7.4, 25 mg/ml asolectin. Subsequent to removal of insoluble material by centrifugation, reconstitution was effected by diluting the solubilized fractions as indicated on the abscissa with Buffer I. Proteoliposomes were assayed for Na$^+$/Ca^{2+} exchange activity as described under "Materials and Methods" (n = 3).

the mixed micellar system to proteoliposomes (e.g., see Reynolds and McCaslin, 1989). Optimal NaCl (480 mM; Figure 4) was also found to be similar to that reported by Miyamoto and Racker (1980). Furthermore, in the absence of NaCl (KCl as a replacement) or exogenous phospholipids, Na^+/Ca^{2+} exchange activity was markedly reduced (data not shown).

Solubilization and reconstitution of the Na^+/Ca^{2+} exchanger by use of cholate under the conditions described here (final concentrations of 1% cholate, 480 mM NaCl, 10 mM Mops-Tris, pH 7.4, 25 mg/ml asolectin with a threefold dilution for reconstitution) resulted in a 32.6-fold increase in specific activity and 202% recovery of total activity (Table 1). Apparent activation of exchange activity may be explained by a number of mechanisms including solubilization of the exchanger from leaky sarcolemmal vesicles followed by reconstitution into sealed proteoliposomes, stimulation by limited proteolysis (Philipson and Nishimoto, 1982), reconstitution into proteoliposomes of high anionic phospholipid content (Hale *et al.*, 1984; Philipson and Nishimoto, 1984; Philipson, 1984; Soldati *et al.*, 1985; Vemuri and Philipson, 1987; Cheon and Reeves, 1988), or possibly by loss of endogenous regulators (Hale *et al.*, 1984; Vemuri and Philipson, 1987).

Given that anionic detergents such as cholate preclude the use of ion exchange chromatography (Renswoude and Kempf, 1984; Hjelmeland and Chrambach, 1984) and may interfere with some lectin affinity chromatography (Lotan *et al.*, 1977; Renswoude and Kempf, 1984), it is desirable to solubilize the
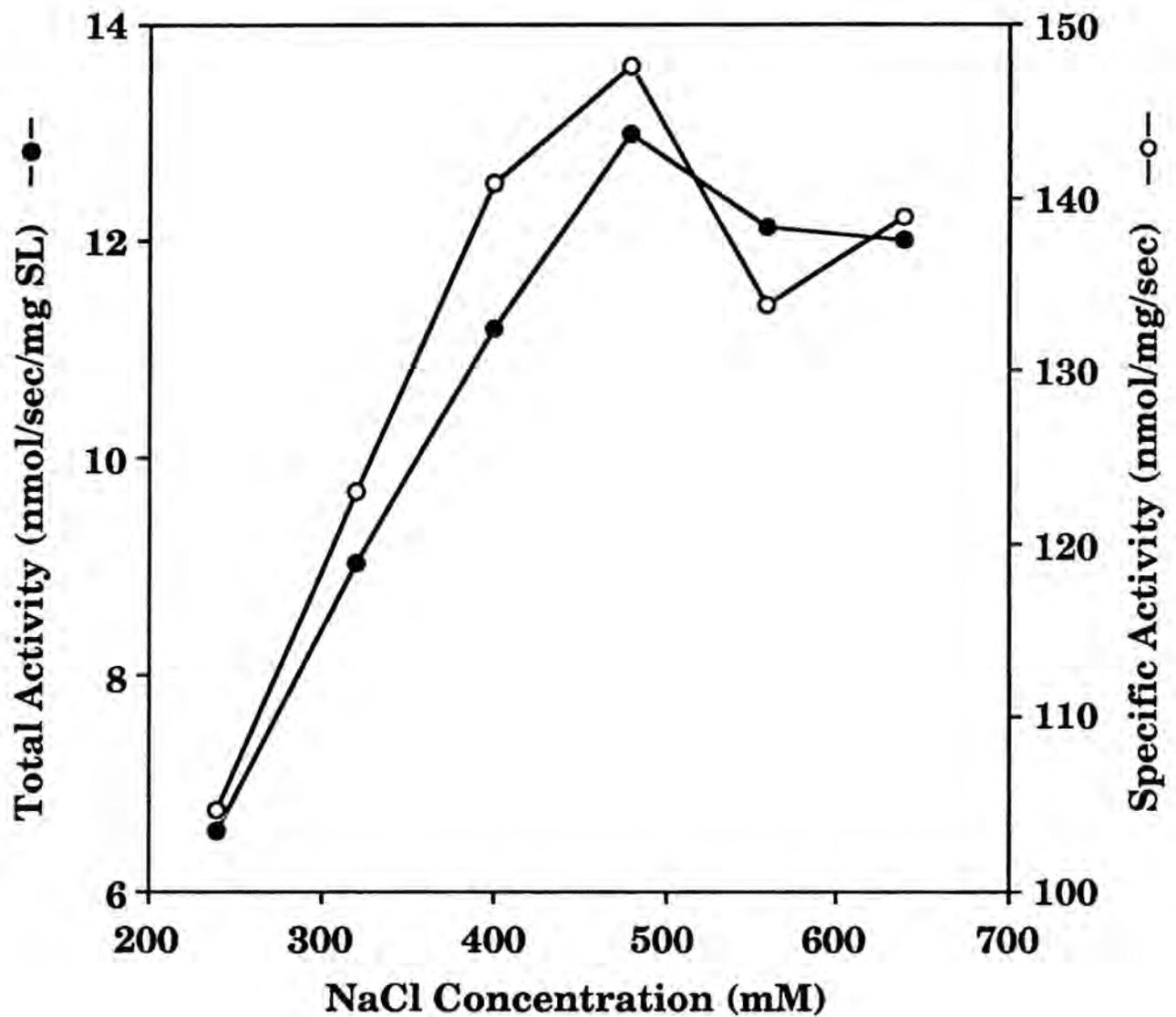
Figure 4. **Recovery of Na+/Ca2+ Exchange Activity Versus NaCl Concentration During Solubilization.** Na+-loaded sarcolemmal vesicles were solubilized with 1% cholate, 10 mM Mops-Tris, pH 7.4, 25 mg/ml asolectin at the indicated NaCl concentrations. Proteoliposomes were formed by a threefold dilution of the solubilized fraction with Buffer I and assayed for Na+/Ca2+ exchange activity as described under "Materials and Methods" (n = 2).

TABLE 1

Na$^+$/Ca^{2+} Exchange Activity in Sarcolemmal and Proteoliposome Preparations.

	Reconstitution	Protein Recovery (μg/mg SL)	Total Activity (nmol/sec/mg SL)	Specific Activity (nmol/mg/sec)
Sarcolemma	—	1000.0	5.2	5.2
Cholate	Dilution	66.1	10.5	169.7
CHAPS	Dialysis	124.0	2.6	21.2
CHAPS	Cholate Dilution	66.1	10.2	159.8
n-octyl-ß-D-glucoside	Cholate Dilution	75.6	10.6	177.1
Decyl-ß-D-maltoside	Cholate Dilution	88.5	7.0	79.3
Triton X-100 (0.188%)	Cholate Dilution	28.5	4.2	148.6
Triton X-100 (1.5%)	Cholate Dilution	38.4	1.0	24.7

Na$^+$-loaded sarcolemmal vesicles were solubilized with either 9 mM CHAPS, 40 mM n-octyl-ß-D-glucoside, 10 mM decyl-ß-D-maltoside, 0.188% Triton X-100 or 1.5% Triton X-100 dissolved in Buffer I or with 1% cholate, reconstituted as indicated, and assayed for Na$^+$/Ca^{2+} exchange activity as described under "Materials and Methods". Values represent the mean of the following number of determinations: sarcolemma, n = 32; cholate, n = 24; CHAPS (dialysis), n = 3; CHAPS (dilution), n = 16; n-octyl-ß-D-glucoside, n = 3; decyl-ß-D-maltoside, n = 3; Triton X-100 (0.188%), n = 2; and Triton X-100 (1.5%), n = 2.

Na$^+$/Ca^{2+} exchanger with nonionic or zwitterionic detergents such as those which have been reported previously (Soldati *et al.*, 1985; Vemuri and Philipson, 1987; Cheon and Reeves, 1988; Cook and Kaupp, 1988; Philipson *et al.*, 1988; Nicoll and Applebury, 1989; Durkin *et al.*, 1990). However, reconstitution by detergent extraction (typically by Amberlite XAD-2 or Bio-Beads SM-2) has resulted in only about a four- to tenfold increase in specific activity (Soldati *et al.*, 1985; Vemuri and Philipson, 1987; Cheon and Reeves, 1988; Philipson *et al.*, 1988; Vemuri and Philipson, 1989). Nicoll and Applebury (1989) reported reconstitution of the rod outer segment Na$^+$/Ca^{2+} exchanger (solubilized with CHAPS) by addition of cholate and asolectin followed by dilution. We found that this combination yielded a 30.7-fold increase in specific activity and a 196% recovery of total activity (Table 1). Conditions for solubilization with CHAPS were initially optimized (9 mM CHAPS; Figure 5) using a previously described dialysis procedure (Cook and Kaupp, 1988) as a means of reconstitution. Recovery of total and specific activity, however, was quite low (Table 1). Reconstitution by cholate dilution provided a 3.9-fold greater recovery of total activity while specific activity was 7.5-fold higher than that obtained with reconstitution by dialysis (Table 1).

At 2% cholate, the amount of asolectin solubilized was found to be less than 50 mg/ml as visually determined after extensive sonication (medium remained turbid). The relationship between light scatter and asolectin concentration suggested that 30-40 mg of the 50 mg phospholipid present could be effectively
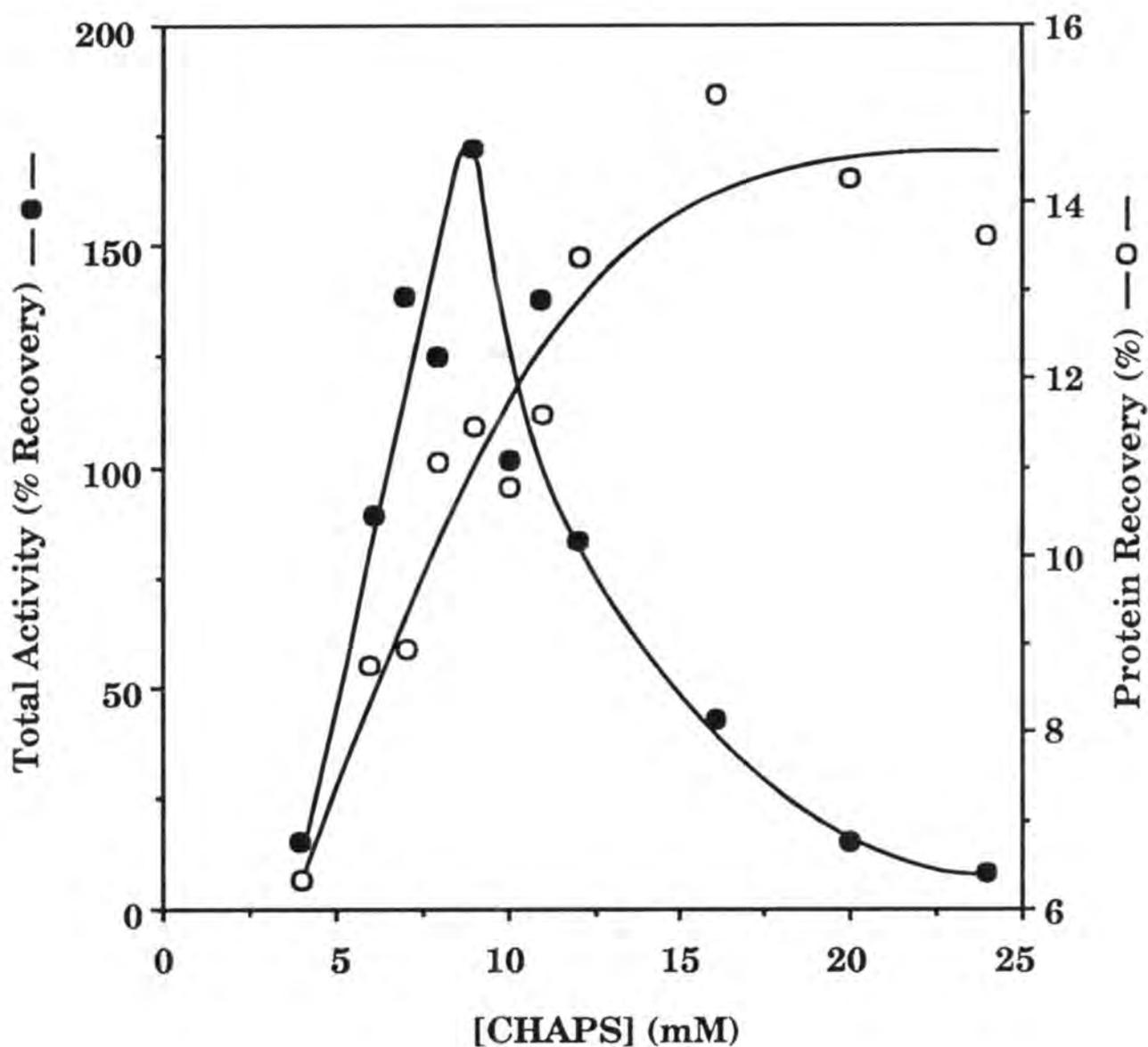
Figure 5. **CHAPS Titration Curve.** Various concentrations of CHAPS, in 160 mM NaCl, 20 mM MOPS-Tris, pH 7.4, were used to solubilize cardiac sarcolemmal vesicles. Proteoliposomes were formed by dialysis (Cook *et al.*, 1986) against 160 mM NaCl, 20 mM Mops-Tris, pH 7.4, 2 mM $CaCl_2$ followed by two changes of the same buffer without $CaCl_2$ at 5°C. Proteoliposomes were recovered by centrifugation and assayed for exchange activity and protein. Na^+/Ca^{2+} exchange activity and protein are expressed as percent of control sarcolemma.

solubilized by 1 ml of 2% cholate in 800 mM NaCl, 10 mM Mops-Tris, pH 7.4 (Figure 6). Prior to the use of cholate dilution for reconstitution of exchange activity solubilized with CHAPS (or other detergents), it was necessary to remove insoluble material from the cholate/phospholipid suspension by centrifugation ("Materials and Methods"). Recovery of total and specific activity from *cholate*-solubilized sarcolemma was unaffected by prior centrifugation of the cholate/phospholipid suspension to remove insoluble material (data not shown).

In addition to CHAPS, several nonionic detergents were screened for their ability to effectively solubilize the exchanger using cholate dilution as a means for reconstitution. The concentrations of the nonionic detergents n-octyl-ß-D-glucoside and decyl-ß-D-maltoside were optimized to yield the greatest recovery of total and specific activity using cholate dilution as a means of reconstitution. Detergent concentrations optimal for solubilization may in some cases affect proteoliposome formation by the cholate dilution technique. The critical micelle concentrations (cmc) for n-octyl-D-glucoside (25 mM; Hjelmeland and Chrambach, 1984) and decyl-D-maltoside (2.2 mM; Alpes *et al.*, 1986) are such that subsequent dilution lowered the detergent concentration below its cmc (important for proteoliposome formation by the cholate dilution technique). Several groups reported use of Triton X-100 (1-1.5%) to solubilize exchange activity from cardiac sarcolemma (Soldati *et al.*, 1985; Vemuri and Philipson, 1987; Cheon and Reeves, 1988; Philipson *et al.*, 1988). However, because the cmc for Triton
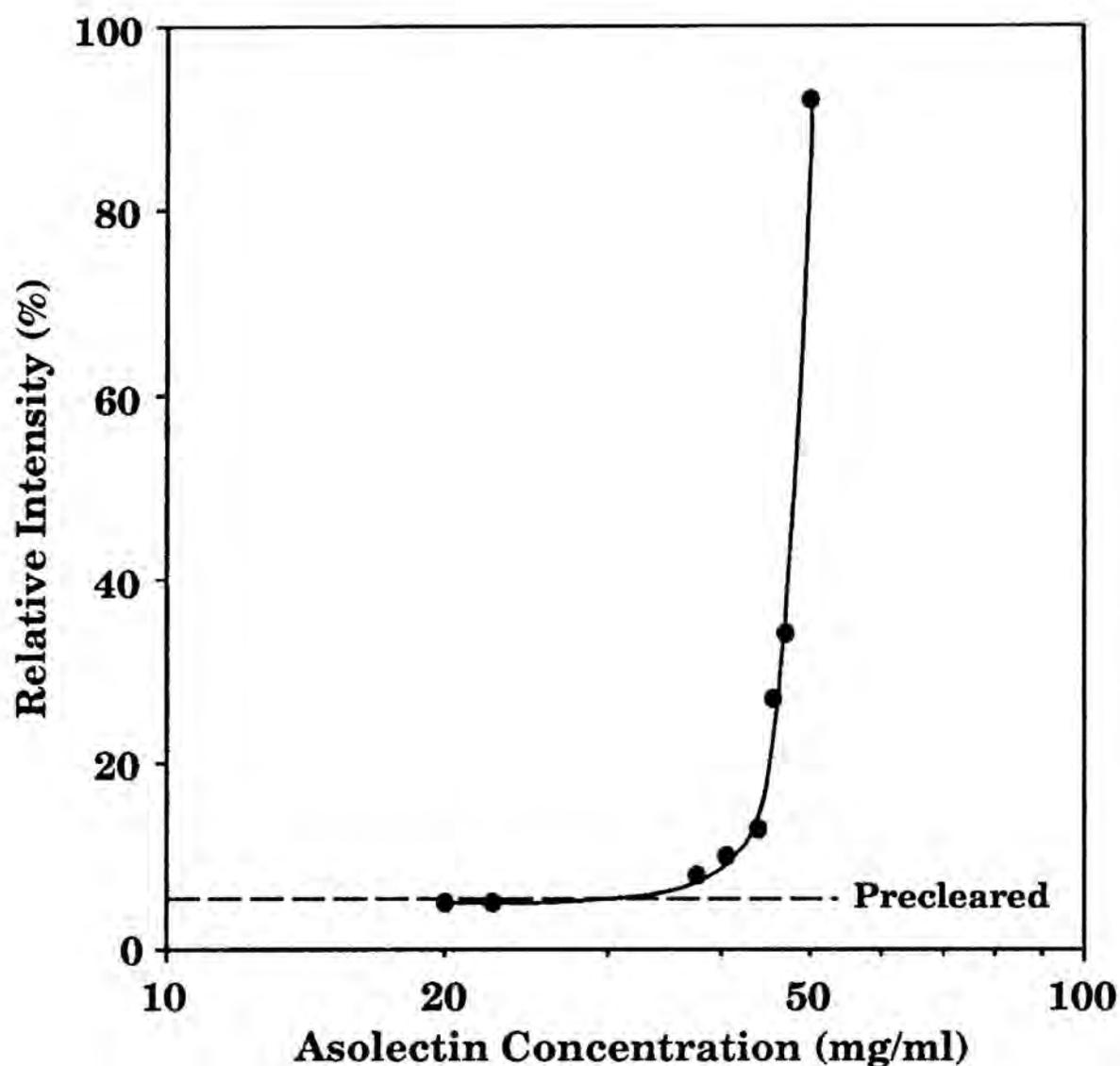
Figure 6. **Solubility of Asolectin in 2% Cholate, 800 mM NaCl, 10 mM Mops-Tris, pH 7.4.** A solution of 2% cholate, 800 mM NaCl, 10 mM Mops-Tris, pH 7.4, 50 mg/ml asolectin was sonicated to uniform suspension with a bath sonifier. The suspension was diluted with 2% cholate, 800 mM NaCl, 10 mM Mops-Tris, pH 7.4 to various asolectin concentrations as indicated. A portion of the suspension was cleared of insoluble material by centrifugation (180,000 x g; 30 min). An Aminco Bowman spectrophotofluorometer was used to determine 90° light scatter at 550 nm. The relative intensity of the precleared fraction was set at 6% (indicated by the broken line). Relative intensity of the 50 mg/ml asolectin solution prior to centrifugation was set at 92%.

X-100 is quite low (0.0188%, Hjelmeland and Chrambach, 1984), solubilization of exchange activity at such high concentrations would undoubtedly result in poor proteoliposome formation by the cholate dilution technique. Therefore, we chose to use two concentrations of Triton X-100 for solubilization: (1) a high concentration (1.5%) such as those reported above, and (2) a low concentration (0.188%) such that subsequent dilution would lower the detergent concentration close to its cmc. As anticipated, proteoliposome formation was greatly reduced when exchange activity was solubilized at 1.5% Triton X-100 and resulted in low recovery of total and specific activities (Table 1). However, solubilization at 0.188% followed by cholate dilution resulted in a 28.6-fold increase in specific activity, but a 19% loss of total activity (Table 1). Interestingly, we found that solubilization of the Na^+/Ca^{2+} exchanger with the detergents listed in Table 1 differed from cholate solubilization in that the presence of exogenous phospholipid during solubilization was no longer required for preservation of activity.

The optimal requirement for asolectin in the cholate medium depended on whether cholate was used for both solubilization and reconstitution or reconstitution alone (solubilization with CHAPS). Optimal concentrations of 15 and 25 mg/ml (asolectin concentrations prior to preclearing) were found for the former and latter cases, respectively (Figure 7). The shift to a higher asolectin concentration with CHAPS is assumed to reflect a higher total detergent concentration at the reconstitution step.
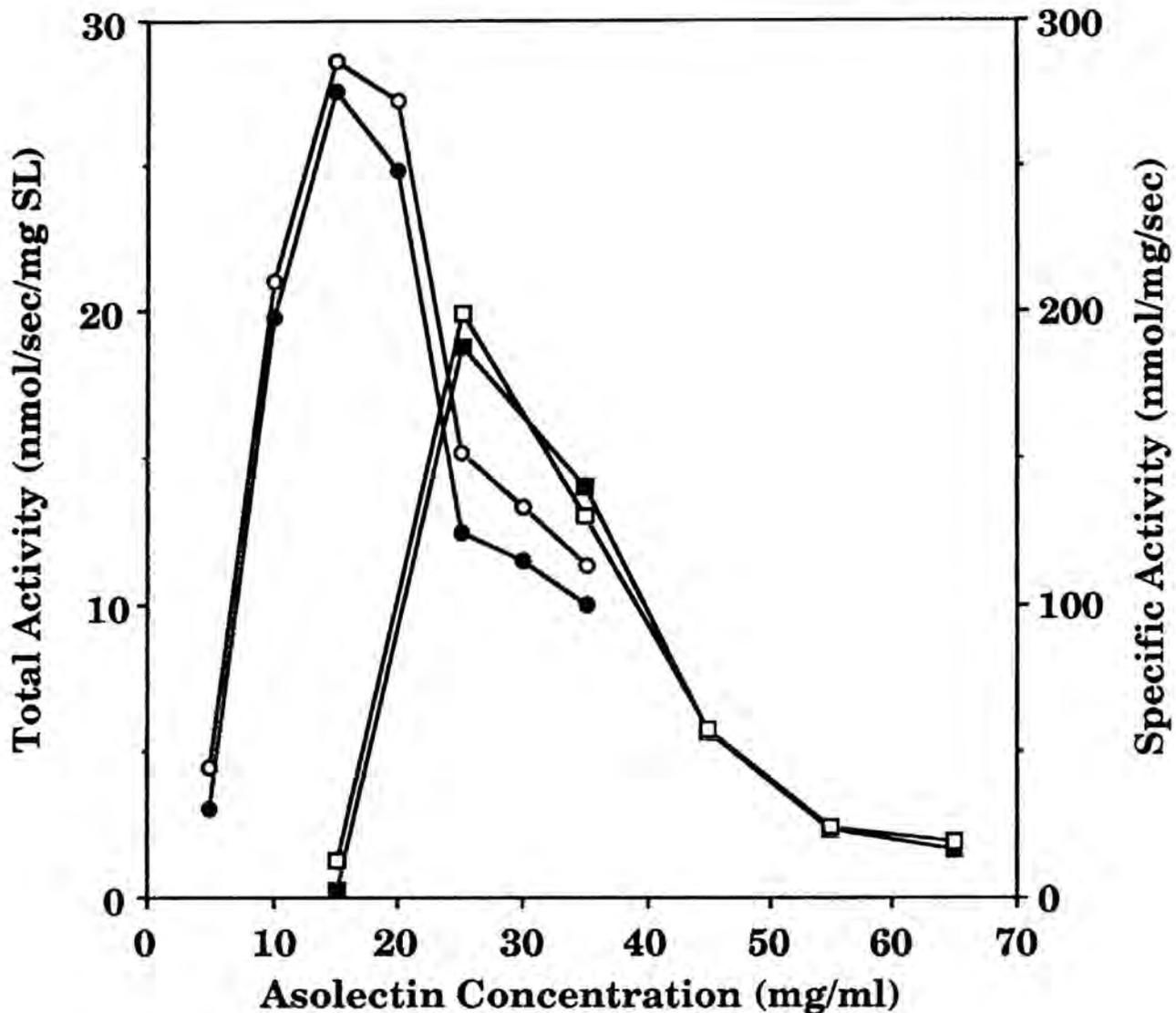
Figure 7. **Recovery of Na+/Ca2+ Exchange Activity versus Asolectin Concentration for Cholate- and CHAPS-solubilized Sarcolemma.** For cholate, Na+-loaded sarcolemmal vesicles were solubilized with 1% cholate, 480 mM NaCl, 10 mM Mops-Tris, pH 7.4 at the specified asolectin concentrations. Insoluble material was removed by centrifugation and protein was reconstituted by a threefold dilution with Buffer I and assayed for Na+/Ca2+ exchange activity (●, total activity; ○, specific activity; n = 3). For CHAPS, Na+-loaded sarcolemmal vesicles were solubilized with 9 mM CHAPS, 160 mM NaCl, 10 mM Mops-Tris, pH 7.4 and reconstituted by cholate dilution as described under "Materials and Methods" where the cholate medium (prior to preclearing) contained varying asolectin concentrations as specified. Proteoliposomes were formed by a threefold dilution of the solubilized fraction with Buffer I and assayed for Na+/Ca2+ exchange activity (■, total activity; □, specific activity; n = 3).

Recovery of exchange activity upon reconstitution was found to be inversely related to the volume/tube centrifuged in the initial collection of the proteoliposomes with maximal recovery achieved at one ml or less per tube (Figure 8). A 50% increase in time or a 25% increase in force of centrifugation resulted in only a slight increase in activity recovered for larger volumes. The difficulty in collecting the proteoliposomes may reflect their low density due to residual detergent. After detergent dilution and centrifugation, the membrane preparation manifested little Na^+/Ca^{2+} exchange activity suggesting leaky or incompletely formed vesicles. In agreement with this interpretation, recovery of activity, following a subsequent wash step to reduce detergent, exhibited no volume dependency for centrifugation and the preparation was characterized by high Na^+/Ca^{2+} exchange activity. While centrifugation of very small volumes is tedious, it does appear necessary for the greatest recovery of activity.

Solubilization with zwitterionic or nonionic detergents allows manipulation of the solubilized proteins for purification without the interference seen with ionic detergents such as cholate or the presence of high concentrations of exogenous phospholipids. However, reconstitution of exchange activity solubilized with these detergents resulted in relatively small increases in specific activity. Cholate and phospholipids, which can be added in combination after protein fractionation procedures, provide a simple and rapid means for reconstitution of the Na^+/Ca^{2+} exchanger, and also provide about a 30-fold increase in specific activity.
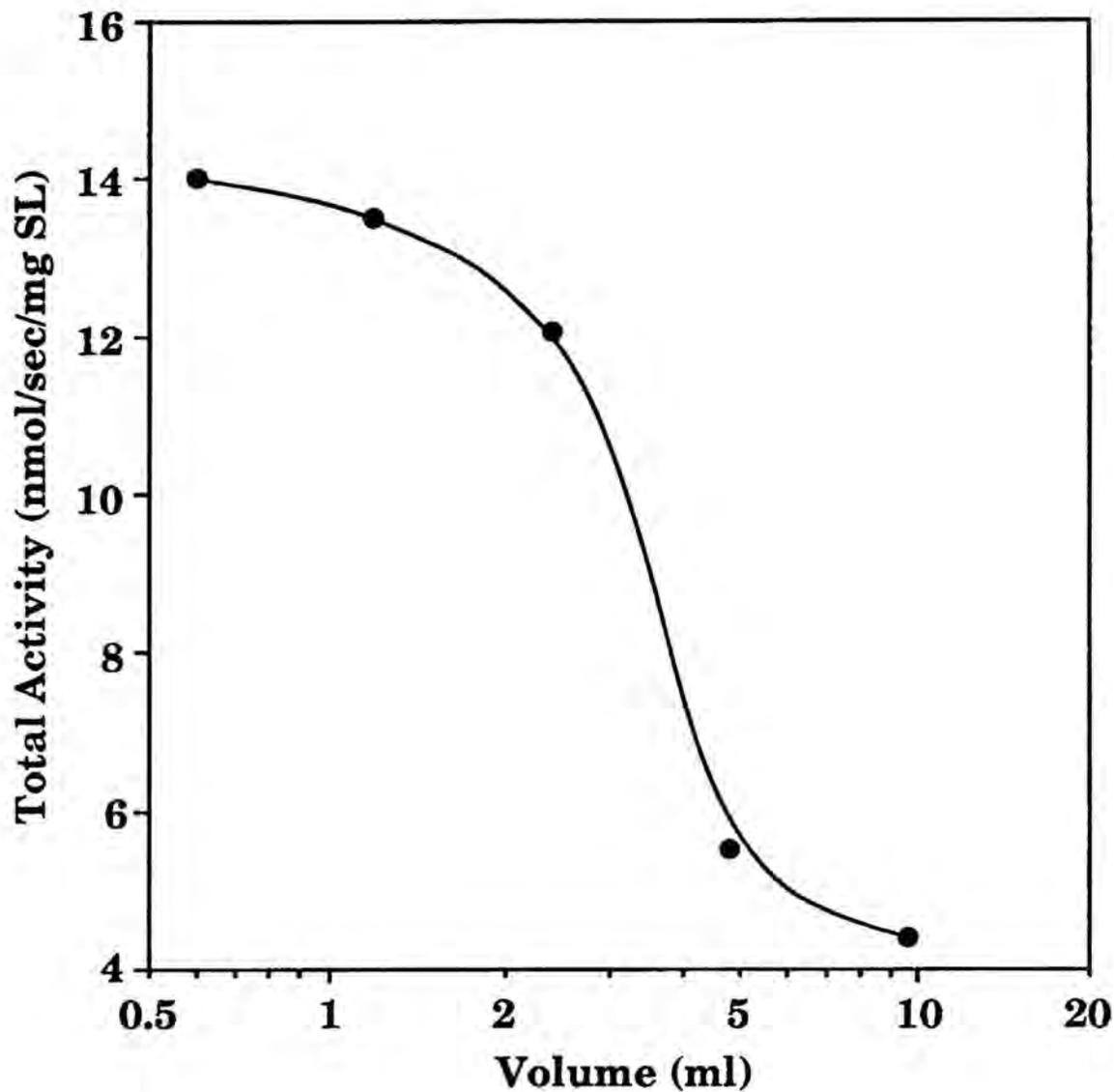
Figure 8. **Recovery of Na+/Ca2+ Exchange Activity Versus the Volume of Sample Centrifuged After Reconstitution.** Na+-loaded sarcolemmal vesicles were solubilized with 9 mM CHAPS, 160 mM NaCl, 10 mM Mops-Tris, pH 7.4. Reconstitution of the solubilized fraction was carried out by cholate dilution as described under "Materials and Methods". Proteoliposomes were pelleted using a Sorval A-125.6 rotor (230,000 x g; 60 min) where the total volume during the initial centrifugation step varied between 0.6 and 9.6 ml. Centrifugation volumes during the washing step were held constant at 10 ml.

We believe that the procedure described above, optimized for the Na^+/Ca^{2+} exchanger, should be applicable to some other integral membrane proteins. Variables that must be tailored for a particular protein include choice and concentration of the detergent used for solubilization. The use of a detergent for solubilization for purification followed by addition of cholate and dilution for reconstitution clearly works best when the first detergent has a high cmc (e.g., CHAPS, n-octyl-ß-D-glucoside, decyl-ß-D-maltoside; Table 1). However, the procedure may be applicable in those cases where sufficient solubilization is achieved at a low concentration of a detergent with a low cmc (e.g., 0.188% Triton X-100; Table 1). Upon selection of the first detergent and its concentration, the amount of lipid added with cholate should be optimized for maximal recovery of the protein upon reconstitution (Figure 7). Finally, the NaCl concentration of the medium immediately prior to dilution was clearly important for reconstitution of the Na^+/Ca^{2+} exchanger (Miyamoto and Racker, 1980; Figure 4). Proteoliposomes were recovered when sodium was replaced by potassium but, as stated above, the recovery of activity was low. Although not systematically studied, the poor recovery upon replacement of sodium probably reflected a stabilization of this particular protein by sodium. In other experiments, the substitution of potassium for sodium in media containing the solubilized exchanger invariably led to substantial loss of Na^+/Ca^{2+} exchange activity (data not shown). Thus, the salt selected to set ionic strength at the reconstitution step might be tailored for the

particular protein under study.

CHAPTER III

Behavior of the Cardiac Na+/Ca^{2+} Exchanger in the Solubilized State: Rapid Purification with Recovery of High Specific Activity

INTRODUCTION

Calcium movements across cardiac sarcolemma (SL) are mediated by Ca^{2+}-channels, a Ca^{2+}-ATPase and the Na^+/Ca^{2+} exchanger (Blaustein, 1988; Figure 1). The Na^+/Ca^{2+} exchanger promotes the coupled movement of sodium and calcium across the cell membrane in a reversible, electrogenic manner (Reeves and Philipson, 1989; Philipson, 1990). The protein responsible for Na^+/Ca^{2+} exchange in canine cardiac tissue has been identified through a combination of purification, immunological and cloning approaches (Philipson *et al.*, 1988; Nicoll *et al.*, 1990).

Structure-function studies of the Na^+/Ca^{2+} exchanger protein will ultimately require optimal strategies for isolating the functionally active protein from tissues and from cell lines overexpressing the protein. As discussed previously (Philipson *et al.*, 1988), strategies for the purification of the Na^+/Ca^{2+} exchanger are currently restricted by several considerations: a) present in cell plasmalemma at a low density; b) lack of selective, high affinity ligands; and c) requirement that solubilized fractions be reconstituted prior to assay of activity.

We have previously reported the utility of solubilizing the cardiac Na^+/Ca^{2+} exchanger with CHAPS followed by the addition of cholate and lipids for reconstitution (Ambesi *et al.*, 1991e). A similar sequence was found to be effective for the rod outer segment (ROS) exchanger (Nicoll and Applebury, 1989). This

allows fractionation of proteins in CHAPS prior to use of the detergent dilution technique (Racker *et al.*, 1979) for reconstitution. However, anomalous behavior of the solubilized cardiac exchanger has been reported for several fractionation procedures. Philipson *et al.* (1988) found that selective elution of the cardiac exchanger from DEAE-Sepharose was highly detergent-dependent. Selective elution was not obtained when solubilization was carried out with CHAPS but was obtained with certain nonionic detergents (e.g., decylmaltoside). Additionally, the cardiac Na^+/Ca^{2+} exchanger, in the presence of CHAPS (Philipson *et al.*, 1988) or high cholate (4%; Durkin *et al.*, 1988), binds well to wheat germ agglutinin (WGA) affinity supports, but little purification is achieved by elution with N-acetyl-D-glucosamine. Rather, purification was effected by elution with nonionic detergents (i.e., Triton X-100, Philipson *et al.*, 1988; polyoxyethylene-9-lauryl ether, Durkin *et al.*, 1988).

Here, we report studies on the behavior of the cardiac Na^+/Ca^{2+} exchanger in the solubilized state with regard to three fractionation procedures. The results led to a sequence for the rapid purification and reconstitution of the exchanger with recovery of specific activities that were approximately 3.7- to 5.6-fold higher than previously reported (Soldati *et al.*,1985; Philipson *et al.* , 1988; Durkin *et al.*, 1991). The purified preparation was characterized by polypeptides of 75, 120 and 140 kilodalton (kDa) upon SDS-PAGE under reducing conditions and of 140 kDa under nonreducing conditions. A minimal turnover number for the purified

preparation was calculated for a monomer of the 140 kDa protein. However, several results suggested the presence of multimeric complexes of the exchanger in the presence of detergent.

EXPERIMENTAL PROCEDURES

Materials

WGA-Sepharose 6MB and DEAE-Sepharose were purchased from Pharmacia LKB Biotechnology Inc.. The TSK 5000 PW high performance liquid chromatography (HPLC) column and Spherogel TM TSK PWH-PRE guard column were obtained from Beckman Instruments Inc. and the Protein-Pak 300 sw HPLC column from Waters. Pepstatin A was purchased from Calbiochem Corporation, leupeptin from Boehringer Mannheim Biochemicals, phenylmethylsulfonyl-fluoride (PMSF) and cholic acid from Serva Biochemicals and CHAPS from Pierce Chemical Corporation. All reagents for SDS-PAGE were electrophoresis grade and obtained from Bio-Rad Laboratories. The Centricell 60 filtration device was purchased from Polysciences, Inc (Warrington, PA) and the Centricon 30 ultrafiltration apparatus was obtained from Amicon (Danvers, MA). $^{45}CaCl_2$ was obtained from New England Nuclear. Soybean phosphatidylcholine Type II-S and Type IV-S were obtained from Sigma Chemical Company and treated as

previously described by Hale *et al.* (1984) and by Cook *et al.* (1986), respectively. All other chemicals were reagent grade.

Purification of the Sarcolemmal Na+/Ca2+ Exchanger

Unless otherwise stated, all procedures were carried out at 4°C. All buffers were freshly prepared and contained the following protease inhibitors: 1μM pepstatin, 1 μM leupeptin and 0.1 mM PMSF. Buffers were filtered (Gelman, 0.2 μm SUPOR-200) immediately prior to use. Sarcolemma enriched preparations from canine ventricle were obtained by modification of the procedure of VanAlstyne *et al.* (1980) as described by Ambesi *et al.* (1991e). Sarcolemmal vesicles were exposed to 160 mM NaCl, 10 mM Mops-Tris, pH 7.4 (Buffer I) for 12-18 hours on ice (Na+–loading) and subsequently subjected to alkaline extraction (Philipson *et al.*, 1987). Briefly, sarcolemmal vesicles (1.5 mg/ml) were diluted 10-fold with ice cold 10 mM 3-(cyclohexylamino)propanesulfonic acid (Caps)-NaOH, pH 12.1, collected by centrifugation (230,000 x g, 30 min) and resuspended in Buffer I at 10 mg of starting sarcolemma/ml. Resuspended membranes were solubilized at 5 mg of starting sarcolemma/ml in 12 mM CHAPS, 160 mM NaCl, 10 mM Mops-Tris, pH 7.4 for 30 min with frequent vortexing. Insoluble material was removed by centrifugation (180,000 x g, 30 min). The supernatant was applied 3 times to a 1.2 x 0.6 cm DEAE-Sepharose column (room temperature) at a flow rate of 0.5 ml/min which had been previously

equilibrated with 100 ml of 9 mM CHAPS, 160 mM NaCl, 10 mM Mops-Tris, pH 7.4, 0.12% phosphatidylcholine (Type IV-S; Buffer II). The column was washed with 15 ml of Buffer II (1 ml/min), and then eluted in a single step with 10 ml of 9 mM CHAPS, 225 mM NaCl, 10 mM Mops-Tris, pH 7.4, 0.12% phosphatidylcholine (Type IV-S) at a flow rate of 1 ml/min. The eluent was collected on ice. The fraction enriched with the Na^+/Ca^{2+} exchanger, eluted from the DEAE-Sepharose, was concentrated to about 500 µl in a Centricell 60 (30,000 Da exclusion limit; 3000 x g, 20 min), transferred to a Centricon 30 and concentrated further (3600 x g, 30 min) to a volume of 100-200 µl. The concentrated fraction was applied to TSK 5000 PW (7.5 x 30 cm) and Protein-Pak 300sw (7.8 x 30 cm) gel filtration HPLC columns linked in tandem which had been equilibrated with Buffer II. The columns were eluted at a flow rate of 0.5 ml/min with Buffer II and 0.8 ml fractions were collected on ice. The fractions enriched in Na^+/Ca^{2+} exchange activity (elution volume, V_e, 15.4 to 19.4 ml) were pooled. The pooled HPLC fractions were then concentrated in a Centricon 30 to 750 µl and diluted with an equal volume of 2% cholate, 0.8 M NaCl, 10 mM Mops-Tris, pH 7.4, 50 mg/ml phosphatidylcholine (Type II-S) which had been sonicated to a uniform suspension with a bath sonifier (Model W185F; Heat Systems-Ultrasonics, Inc.) and cleared of insoluble material by centrifugation (180,000 x g, 30 min; Buffer III). This was then applied to a 1.0 ml bed volume of WGA-Sepharose which had been equilibrated with 100 ml of Buffer IV (Buffer II diluted with an equal volume of Buffer III) and incubated for

30 min with gentle agitation at room temperature. The nonbound fraction was collected and the WGA-Sepharose was washed with four 1.0 ml aliquotes of Buffer IV. All fractions were collected on ice, reconstituted separately and then pooled. This constituted the purified Na+/Ca2+ exchanger preparation.

Protein Reconstitution

Reconstitution of solubilized proteins was performed by modifications of the Miyamoto and Racker (1980) procedure as described by Ambesi *et al.* (1991e). Briefly, samples for reconstitution were adjusted to final concentrations of 1% cholic acid, 4.5 mM CHAPS, 480 mM NaCl, 10 mM Mops-Tris, pH 7.4, 25 mg/ml phosphatidylcholine (Type II-S) by a 2-fold dilution of CHAPS-solubilized sample with Buffer III. Note that the nonbound and wash fractions collected off the WGA-Sepharose were already adjusted to these concentrations. After incubation on ice for 5 min, proteoliposomes were formed by a 3.2-fold dilution with Buffer I, collected by centrifugation (230,000 x g, 60 min), then washed once by resuspension with Buffer I and centrifugation as above. Proteoliposomes were resuspended in 100-300 µl of Buffer I and used for assays of activity and protein, SDS-PAGE, immunoreactivity and the generation of polyclonal antibodies.

Assay for Na+/Ca2+ Exchanger

Na+/Ca2+ exchange assays were carried out according to the procedure of Reeves and Sutko (1983) with slight modifications. Briefly, for Na^+_i-dependent $^{45}Ca^{2+}$ uptake assays, vesicles were either preloaded with Buffer I for 12-18 hours on ice (sarcolemma preparations) or formed in the presence of NaCl (proteoliposomes) trapping Na+ inside. Na+/Ca2+ exchange activity was measured by rapidly diluting 5 µl of Na+-loaded vesicles into 245 µl of uptake medium containing 160 mM KCl, 10 mM Mops-Tris, pH 7.4, 0.5 µM valinomycin (added as a 0.1 mM stock solution in absolute ethanol), 40 µM $^{45}CaCl_2$ (specific activity 150-250 cpm/pmol) at 37°C. The reaction was terminated after 1 sec by the addition of 5 ml of ice-cold stop solution (160 mM KCl, 0.1 mM EGTA, 10 mM Mops-Tris, pH 7.4). Timing was carried out using a metronome. The vesicles were filtered (Millipore, Type HA 0.45 µm) and the filter washed with 3 x 5 ml of the ice-cold stop solution. The $^{45}Ca^{2+}$ content on the filters was determined by liquid scintillation techniques. Values for $^{45}Ca^{2+}$ uptake were corrected by subtracting the amount of uptake in the absence of a Na+-gradient (reaction media contained 160 mM NaCl instead of 160 mM KCl).

Electrophoresis and Western Blotting

SDS-PAGE was performed as described by Laemmli (1970) using a linear

4-12% gradient resolving gel and 4% stacking gel. Reconstituted samples were first precipitated with acetone (–20°C) and extracted with diethyl ether to remove excess lipid. Sample buffer contained the following protease inhibitors: 1 µM pepstatin, 1 µM leupeptin, 0.1 mM PMSF and 0.1 mM EDTA. Solubilization (2% SDS; 0.5% Triton X-100) was carried out in the presence of either 100 mM dithiothreitol (DTT; reducing conditions) or 10 mM N-ethylmaleimide (NEM; nonreducing). Samples were heated to 95–98°C for 3 min. Proteins were visualized by silver staining according to the method of Heukeshoven and Dernick (1985). For immunoblots, proteins from SDS-PAGE were transferred onto Immobilon-P (Millipore; polyvinylidene difluoride) for 30 min at 30 volts followed by an additional 30 min at 100 volts using a Bio-Rad Trans-Blot Cell (plate electrodes). Blots were blocked in Tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris-Cl, pH 7.4) containing 5% nonfat milk and 2% fetal bovine serum for 1 hour at 37°C, washed with three changes (5 min each) of TBS, 0.1% BSA and incubated 2 hours at room temperature with antiserum diluted 500-fold into TBS containing 0.05% Tween-20. Blots were then washed as above and immunoreactions were detected by autoradiography using [125I]protein A (Huynh *et al.*, 1985). Briefly, immunoblots were washed as above, followed by an additional 5 min wash in TBS, 20% fetal bovine serum and incubated 1 hour at room temperature in TBS, 20% fetal bovine serum containing 0.09 µCi [125I]protein A/ml. Blots were then washed as follows: (1) 10 min in TBS, (2) 10 min in TBS, 0.1% Triton X-100 followed by (3) 10

min in TBS. Subsequently, blots were dried and exposed to Kodak X-OMAT AR autoradiographic film for 1 to 7 days.

Generation of Polyclonal Antibodies

The Na$^+$/Ca^{2+} exchanger was purified and reconstituted as described above. Between 50 and 75 µg of purified Na$^+$/Ca^{2+} exchanger were emulsified with Freund's complete adjuvant and injected into the hind leg muscles and subcutaneously at multiple sites on the back of a male New Zealand white rabbit. Four weeks after primary immunization, the procedure was repeated except that Freund's incomplete adjuvant was used. Antiserum was collected ten days after boosting.

Enzyme-Linked Immunosorbent Assay (ELISA)

Microtitration plates (polyvinyl chloride; 96-well plates) were coated 18 hours at 4°C with either 0.25, 0.50 or 1.00 µg of canine cardiac sarcolemma diluted into 100 µl of phosphate-buffered saline (PBS). The plates were washed 3X with PBS, 0.1% BSA and blocked 1 hour at 37°C with 250 µl PBS, 3% BSA. The plates were washed 3X with PBS, 0.05% Tween-20 and incubated 1.5 hours at 37°C with antiserum diluted (various dilutions) into 80 µl PBS, 0.05% Tween-20, 1% BSA. The plates were washed 7X with PBS, 0.05% Tween-20 and incubated 1.0 hour at

room temperature with goat anti-rabbit IgG-horseradish peroxidase conjugate diluted (1000-fold) into 80 μl PBS, 0.05% Tween-20, 1% BSA. The plates were washed 7X with PBS, 0.05% Tween-20 and incubated 10 min at room temperature with 100 μl 0.03% ABTS [2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid), diammonium salt], 0.003% H_2O_2 in 0.05 M citrate buffer, pH 4.0 and read at 414 nm in an automated spectrophotometric microtitration plate reader.

Protein Assays

Protein was determined by the method of Lowry *et al.* (1951) for sarcolemma preparations and by the method of Kaplan and Pedersen (1985) for reconstituted proteoliposome preparations. Bovine serum albumin was used as the standard.

RESULTS

The procedure developed for purification of the canine cardiac sarcolemmal Na^+/Ca^{2+} exchanger is summarized in Figure 9.

Alkaline Extraction and Solubilization

The use of alkaline extraction of sarcolemmal membranes, as described by Philipson *et al.* (1987), provides a quick and simple means for enriching Na^+/Ca^{2+}

Canine Cardiac Sarcolemmal Preparation

↓

Load Vesicles with 160 mM NaCl, 10 mM Mops-Tris, pH 7.4

↓

Wash Membranes at pH 12.1

↓

Solubilize with 12 mM CHAPS

↓

DEAE-Sepharose Chromatography

↓

Gel Filtration HPLC

↓

Wheat Germ Agglutinin-Sepharose Chromatography

↓

Reconstitute into Proteoliposomes by Cholate Dilution
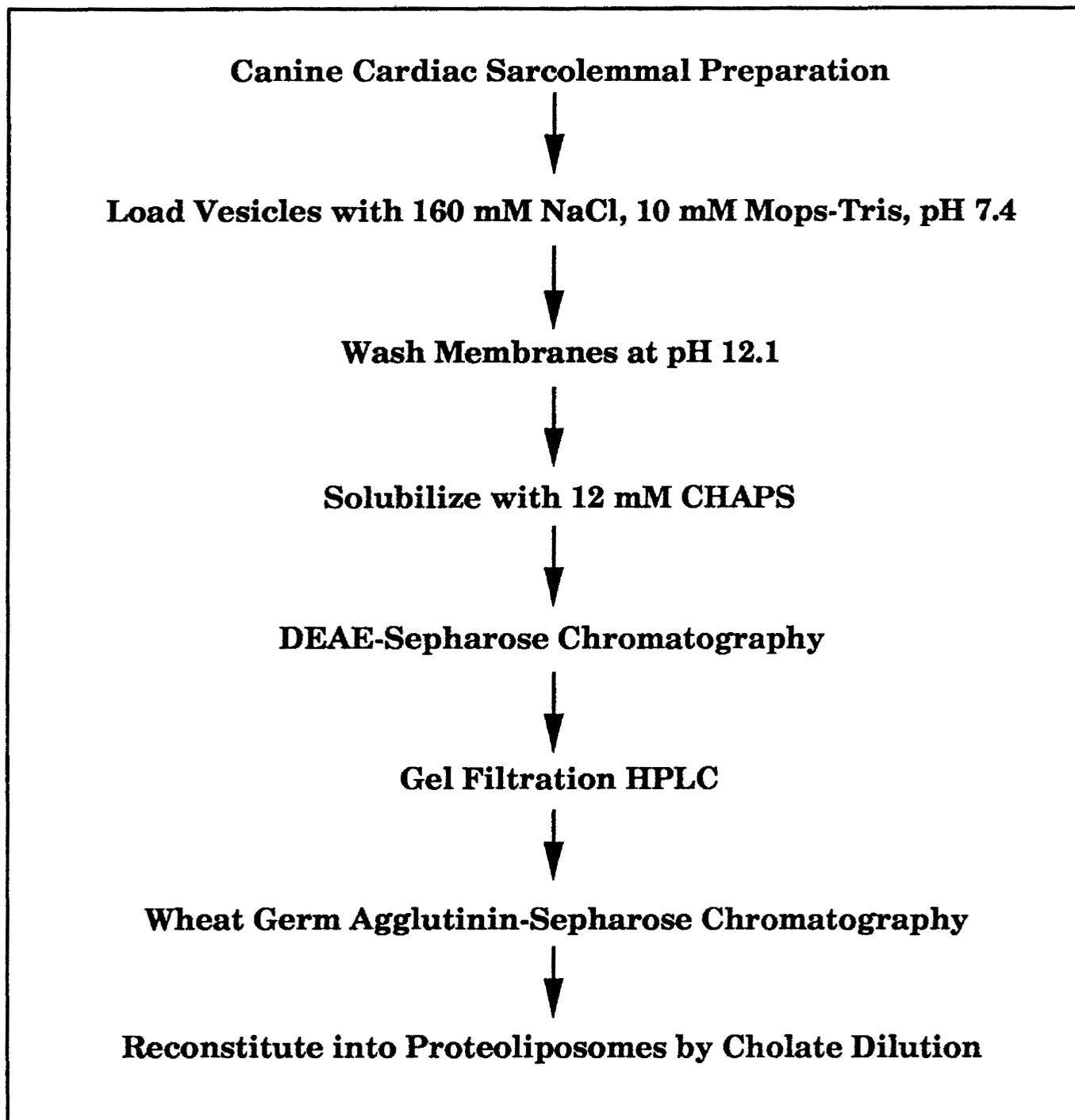
Figure 9. **Purification Scheme for the Canine Cardiac Na^+/Ca^{2+} Exchanger.** All steps were carried out in the presence of protease inhibitors (1 µM pepstatin, 1 µM leupeptin and 0.1 mM PMSF).

exchange activity. Alkaline extraction of sarcolemmal membranes at pH 12.1 provided a 3-fold enrichment of Na^+/Ca^{2+} exchange activity over control proteoliposomes (87–fold over SL) with 72% recovery of activity and a specific activity of 452 nmol/mg/sec (n = 30; Table 2).

Previous studies (Ambesi *et al.*, 1991e) have shown that the concentration of CHAPS required for optimal solubilization and recovery of Na^+/Ca^{2+} exchange activity is 9 mM for a protein concentration of 0.75 mg/ml. However, solubilization at low protein concentrations (0.75 mg/ml) results in excessively large volumes upon scale-up for purification of the exchanger. In order to keep volumes at a minimum, we chose to solubilize at a higher protein concentration. To accomplish this, alkaline extracted membranes were solubilized at 5 mg of starting sarcolemma/ml. The concentration of CHAPS required for optimal solubilization and recovery of exchange activity shifted to 12 mM under these conditions. The correlation between detergent concentration required for solubilization and protein concentration has been previously noted (Hjelmeland and Chrambach, 1984).

DEAE-Sepharose Chromatography

Solubilized membranes were applied to a DEAE-Sepharose column as indicated under "Experimental Procedures". At 160 mM NaCl, greater than 95% of the applied Na^+/Ca^{2+} exchange activity and about 50% of the solubilized protein

TABLE 2

Purification of the Cardiac Na$^+$/Ca^{2+} Exchanger

Fraction	Protein Recovery (%)	Total Activity (nmol/sec/mg SL)	Specific Activity (nmol/mg/sec)	Purification Factor
Sarcolemma	100.00	5.2	5.2	–
Control Proteoliposomes[a]	4.75	7.1	152.3	1.0
Alkaline Extraction	0.92	5.1	451.7	3.0
DEAE-Sepharose	0.21	3.5	1220.5	8.0
Gel Filtration	0.07	1.5	2917.5	19.2
WGA-Sepharose	0.02	0.7	3765.6	24.7

[a] Sarcolemmal proteins were solubilized in CHAPS and reconstituted as previously described (Ambesi *et al.*, 1991e). Since total exchange activity is stimulated upon solubilization and reconstitution (Hale *et al.*, 1984; Soldati *et al.*, 1985; Philipson *et al.*, 1987), specific activities in subsequent steps are compared with control proteoliposomes rather than with sarcolemma. Note that alkaline extraction preceded solubilization whereas the last three steps followed solubilization (see text).

was bound (Figure 10). Increasing ionic strength to 225 mM NaCl in a single step eluted 82% of the applied Na^+/Ca^{2+} exchange activity and 17% of the solubilized protein when compared to paired controls. This fraction was used for further purification of the Na^+/Ca^{2+} exchanger. Subsequent to reconstitution, specific activity was increased to 1221 nmol/mg/sec, an enrichment of 235-fold over that of sarcolemma, with 69% recovery of activity (n = 8; Table 2). Increasing ionic strength from 225 mM to 1 M NaCl eluted additional protein (33%) and Na^+/Ca^{2+} exchange activity (14% of the applied activity; Figure 10). Identification of the proteins involved with Na^+/Ca^{2+} exchange at this stage in the purification was not possible due to the presence of numerous proteins.

Gel Filtration HPLC

The Na^+/Ca^{2+} exchanger enriched fraction eluted from DEAE-Sepharose with 225 mM NaCl was concentrated by ultrafiltration and subjected to gel filtration HPLC. A single activity peak eluted at a relative molecular weight of 370 kDa (V_e = 16.2 ml; Figure 11), as determined by molecular weight standards run under identical column conditions (Figure 11, *upper inset*), and correlated well with the elution of a single major absorbance peak detected at $V_e \sim 16.5$ ml. The absorbance peak detected at $V_e \sim 16.5$ ml provided an indirect means for predicting the absolute amount of total exchange activity recovered in the purified fraction subsequent to WGA–Sepharose chromatography and reconstitution (Figure 11,
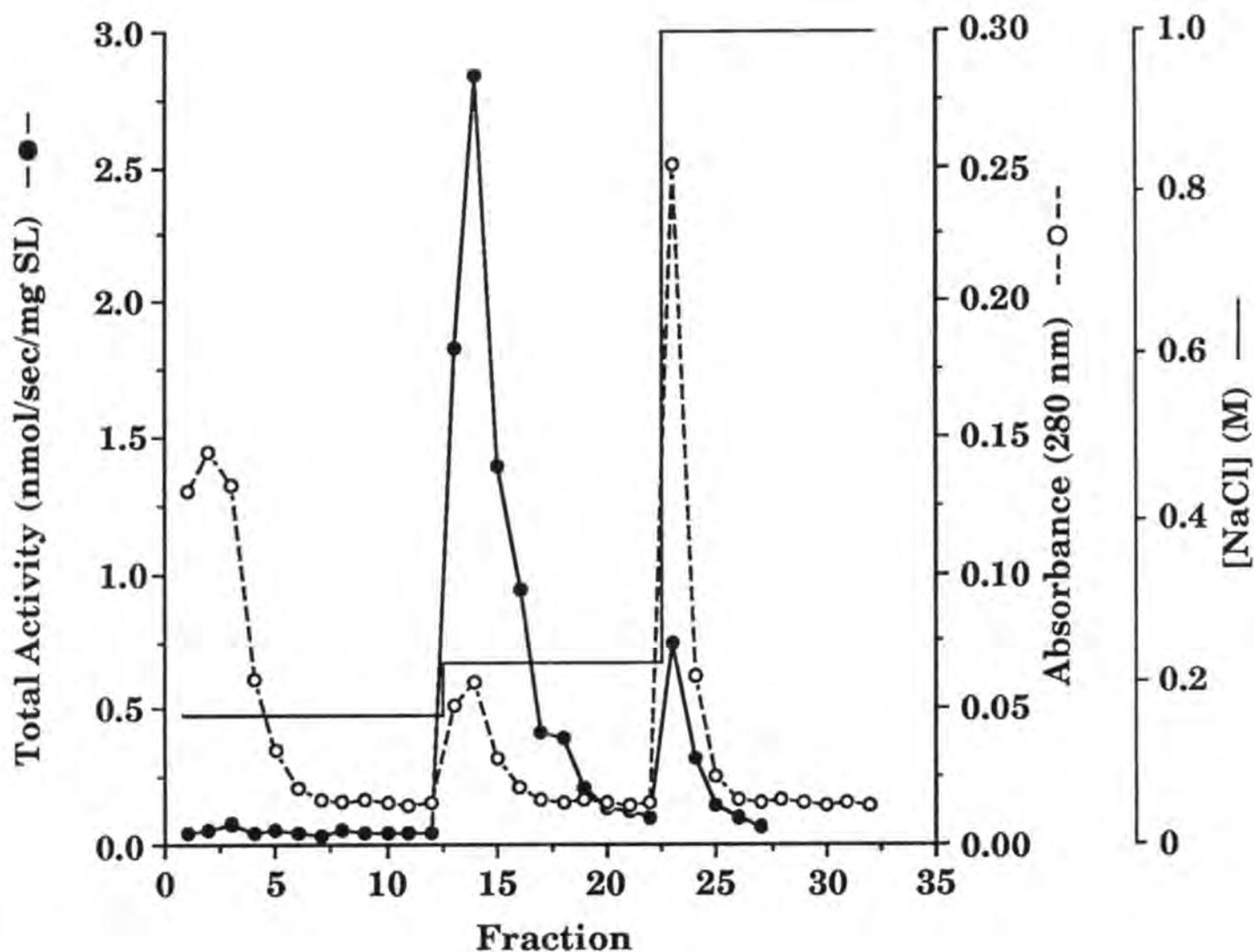
Figure 10. **DEAE-Sepharose Chromatography of Alkaline Extracted, CHAPS-solubilized Sarcolemma.** Sarcolemmal membranes (10 mg) were alkaline-extracted, solubilized with CHAPS and applied to a DEAE-Sepharose column as described under "Experimental Procedures". The column was washed with 10 ml of Buffer II and exchange activity and protein were eluted by increasing the concentration of NaCl. Fractions (1.0 ml) were collected, reconstituted into proteoliposomes and assayed for exchange activity and protein. Absorbance at 280 nm, ---o---; NaCl concentration, ---; total Na+/Ca2+ exchange activity, -•-.

Figure 11.   **Gel Filtration HPLC of the DEAE-enriched, Na$^+$/Ca^{2+} Exchanger Fraction.** Sarcolemmal membranes (40 mg) were subjected to alkaline extraction, solubilization and DEAE-Sepharose chromatography ("Experimental Procedures"). The fraction enriched in Na$^+$/Ca^{2+} exchange activity eluted from the DEAE-Sepharose column was concentrated by ultrafiltration and applied to TSK 5000 PW and Protein-Pak 300 sw gel filtration HPLC columns linked in tandem. Exchange activity was eluted with Buffer II at 5°C and a flow rate of 0.5 ml/min. Fractions (0.8 ml) were collected on ice and reconstituted as described under "Experimental Procedures". Void and total column volumes are indicated by V_o and V_t, respectively. Absorbance at 280 nm (0.1 AUFS), ---; total Na$^+$/Ca^{2+} exchange activity, -•-; protein recovery, -o-. *Upper inset*: Gel filtration calibration standards (Pharmacia) were run under identical column conditions as the DEAE-enriched, Na$^+$/Ca^{2+} exchanger fraction. Molecular mass is plotted as a function of the elution volume corresponding to peak absorbance. Arrow indicates elution volume corresponding to peak recovery of Na$^+$/Ca^{2+} exchange activity. Gel filtration calibration standards: thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa. *Lower inset*: Absolute amount of Na$^+$/Ca^{2+} exchange activity (nmol/sec) recovered subsequent to purification (following WGA–Sepharose chromatography and reconstitution) as a function of absorbance (280 nm) observed for the peak detected at $V_e \sim 16.5$ ml.
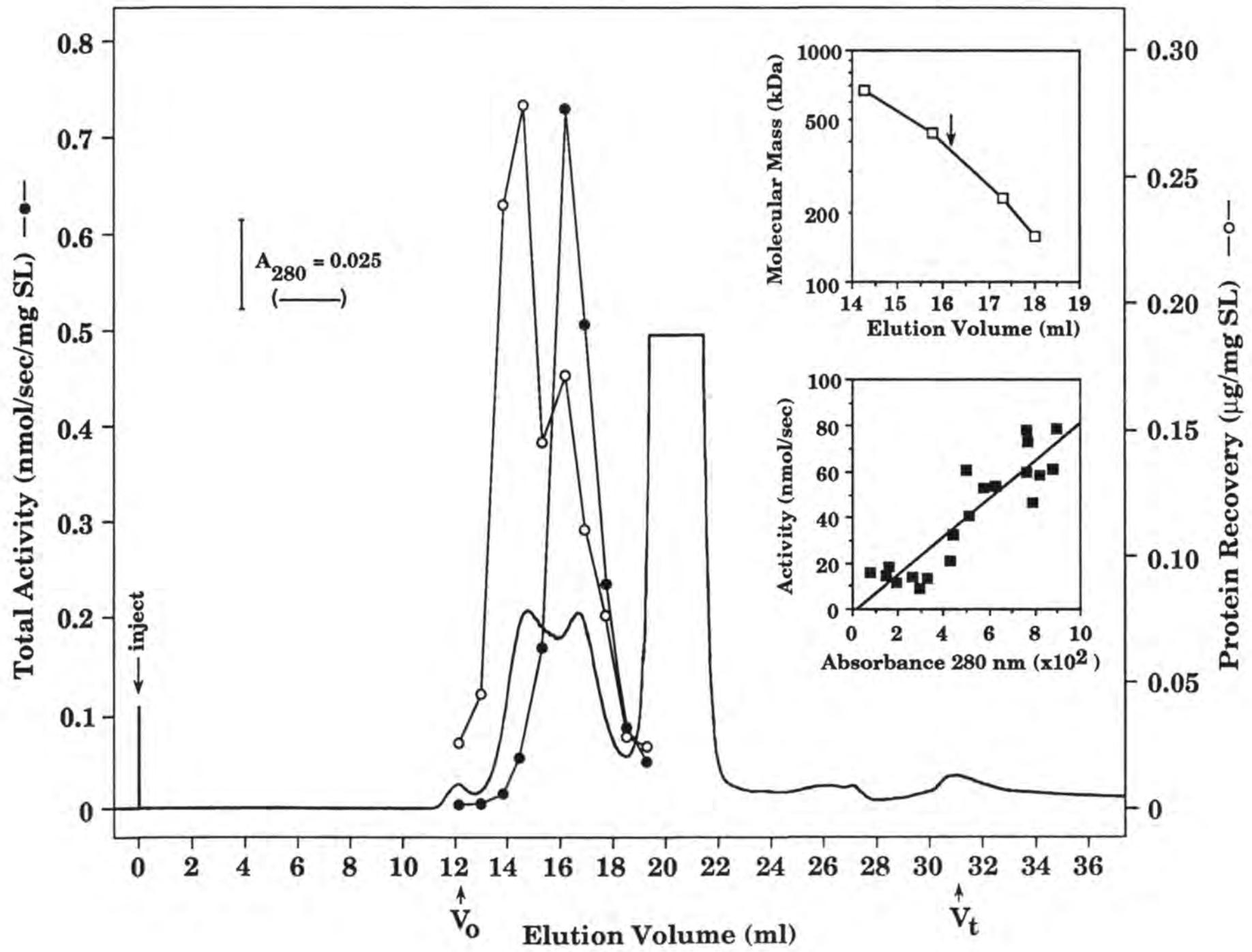
lower inset). Fractions corresponding to peak Na^+/Ca^{2+} exchange activity were subjected to SDS-PAGE and found to be enriched in proteins of 75, 120 and 140 kDa. Although these three proteins were found to correlate well with exchange activity and the major absorbance peak detected at V_e~16.5 ml, a number of minor bands were also detected. Reconstitution of fractions eluted between 15.4 ml and 17.8 ml provided an additional 2.4-fold enrichment (561-fold over sarcolemma) with a specific activity of 2918 nmol/mg/sec (n = 7) and 21% recovery of activity (Table 2). The fractions collected between 15.4 ml and 19.4 ml were used for further purification of the Na^+/Ca^{2+} exchanger.

Curiously, when fractions collected between 19.4 ml and 22 ml (largest absorbance peak detected) were subjected to reducing SDS–PAGE, very little or no protein was detected. We chose to further investigate the source of this large absorbance peak (V_e~20 ml). In a typical purification run, Na^+/Ca^{2+} exchange activity is eluted from DEAE–Sepharose with 10 ml of 9 mM CHAPS, 225 mM NaCl, 10 mM Mops–Tris, pH 7.4, 0.12% phosphatidylcholine and concentrated approximately 100–fold as described under "Experimental Procedures". In a similar manner, 10 ml of DEAE elution buffer (no protein) was concentrated ~100–fold and run under identical HPLC conditions. Two peaks were detected. A small absorbance peak was detected at V_e~12 ml (void volume, V_o) and a large absorbance peak at V_e~20 ml, similar to that seen in Figure 11. We attribute this peak to light scatter by detergent/lipid micelles.

WGA-Sepharose Chromatography

Fractions enriched in Na^+/Ca^{2+} exchange activity, collected from the gel filtration HPLC columns (V_e = 15.4 to 19.4 ml), were pooled, concentrated by ultrafiltration to 750 µl, diluted with an equal volume of a medium containing high salt, cholate and lipids (Buffer III) and incubated with WGA-Sepharose ("Experimental Procedures"). The nonbound and wash fractions were reconstituted and assayed for Na^+/Ca^{2+} exchange activity and protein and analyzed by SDS-PAGE. When compared to paired controls, 67% of the applied activity was recovered with 85% of the recovered activity nonbound and 15% eluted with 0.4 M N-acetyl-D-glucosamine (n = 2). The first two 1.0 ml nonbound fractions collected off the WGA-Sepharose were most highly enriched in Na^+/Ca^{2+} exchange activity and provided a 724-fold enrichment over sarcolemma with a specific activity of 3766 nmol/mg/sec (n = 5; Table 2). Overall recovery of activity was 10% compared to control proteoliposomes. SDS-PAGE revealed three major bands at 75, 120 and 140 kDa (Figure 12, *lane 3*) and trace minor proteins under reducing conditions. Under nonreducing conditions (Figure 12, *lane 4*), the 75 and 120 kDa proteins were not detected and one major band centered at 140 kDa was observed. A diffuse band at ~250–300 kDa was detected under both reducing and nonreducing conditions on silver stained gels.

Figure 12. **Silver Stain and Western Blot Analysis of Sarcolemmal Proteins and the Purified Na+/Ca2+ Exchange Preparation.** Sarcolemmal membranes and the purified Na+/Ca2+ exchange preparation were subjected to reducing and nonreducing SDS–PAGE as described under "Experimental Procedures". Proteins were either silver stained (*lanes 1–4*) or electrotransferred to Immobilon–P for western blot analysis (*lanes 5–8*). Immunoreactions of blotted proteins (500–fold dilution of antiserum) were detected by autoradiography using [125I]protein A. Molecular mass markers are shown on left. Silver Stain: *lanes 1* and *2*, 40 µg sarcolemmal membranes under reducing and nonreducing conditions, respectively; *lanes 3* and *4*, purified Na+/Ca2+ exchange preparation (from 9 mg SL) under reducing and nonreducing conditions, respectively. Western Blot: *lanes 5* and *6*, 10 µg sarcolemmal membranes under reducing and nonreducing conditions, respectively; *lanes 7* and *8*, purified Na+/Ca2+ exchange preparation (from 1 mg SL) under reducing and nonreducing conditions, respectively.

Silver Stain   **Western Blot**

kDa

200 —

116 —

97 —

66 —

45 —

31 —

21 —
14 —

1   2   3   4   5   6   7   8

kDa

←140
←120

←75
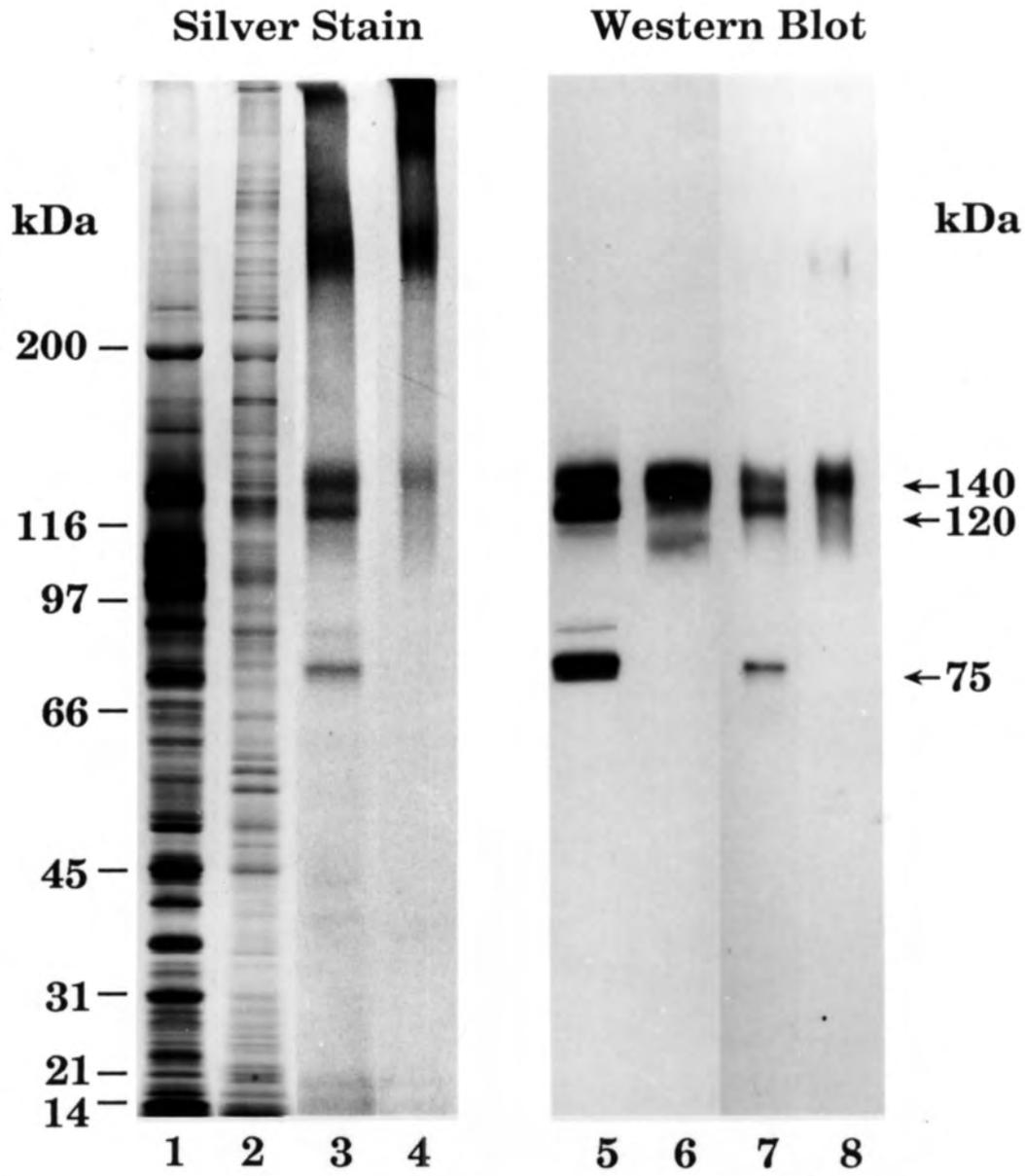
Immunological Studies

Polyclonal antibodies were developed against the reconstituted, purified Na$^+$/Ca^{2+} exchanger preparation as described under "Experimental Procedures". The antiserum was initially tested for the presence of sarcolemma-specific antibodies by ELISA (plates coated with either 0.25, 0.50 or 1.00 μg sarcolemmal protein per well). Immunoreactions were detected at each protein value. As expected, antibody sensitivity (antibody dilution giving half maximal saturation) decreased with decreasing sarcolemmal protein (Figure 13).

These antibodies immunoprecipitate 97% of the Na$^+$/Ca^{2+} exchange activity from detergent-solubilized sarcolemma (Ambesi *et al.*, 1991c). Figure 12 shows the reaction of these antibodies with the purified Na$^+$/Ca^{2+} exchanger preparation under reducing (*lane 7*) and nonreducing (*lane 8*) conditions. Under reducing conditions, strongest reactions occurred at 75, 120 and 140 kDa. With longer exposures, minor reactions were detected at 85, 114 and ~250–300 kDa. Under nonreducing conditions, the strongest reaction was centered at 140 kDa with minor reactions at 114 and ~250–300 kDa. The immunoreaction seen at ~250–300 kDa was strongest under nonreducing conditions. Curiously, this band immunoblots relatively weakly (Figure 12, *lanes 7* and *8*), yet stains heavily with silver (Figure 12, *lanes 3* and *4*). This may be the result of poor transfer from gel to Immobilon–P. Whether this band is related to Na$^+$/Ca^{2+} exchange or not is unclear, although it may represent a dimer of the 140 kDa protein. The detection
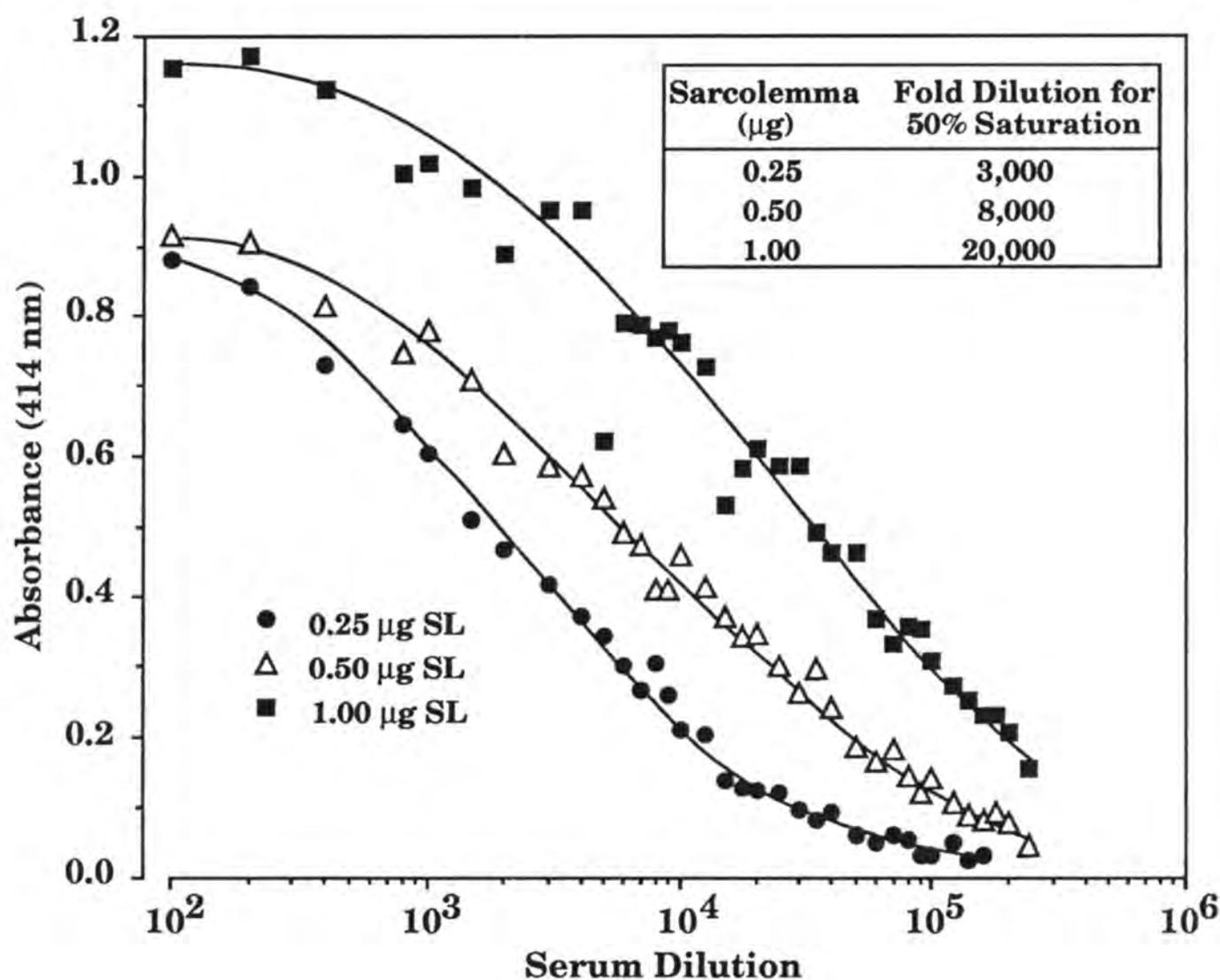
Figure 13.   **Enzyme-Linked Immunosorbent Assay.**   Microtitration plates (96-well) were coated with 0.25, 0.50 or 1.00 µg of sarcolemma and incubated with antiserum diluted 100- to 240,000-fold ("Experimental Procedures").   Immunoreactions were detected with horseradish peroxidase-conjugated anti-rabbit IgG; color development was quantitated spectrophotometrically.

of multimers of certain integral membrane proteins upon SDS-PAGE has been well documented (e.g., rhodopsin; see Molday and Molday, 1979; De Grip *et al.*, 1980; Weiss *et al.*, 1988).

Figure 12 also shows immunoreactions of sarcolemmal proteins under reducing (*lane 5*) and nonreducing (*lane 6*) conditions. Major reactions under reducing conditions were nearly identical to those seen in the purified exchanger preparation and occurred at 75, 120 and 140 kDa with minor reactions seen at 85 and 114 kDa. Immunoreaction in the region of ~250–300 kDa, however, was not detected. Under nonreducing conditions, the major reaction was centered at 140 kDa.

DISCUSSION

We report here fractionation procedures for purifying the cardiac sarcolemmal Na^+/Ca^{2+} exchanger and the development of polyclonal antibodies which immunoprecipitate exchange activity. Steps in the purification scheme include alkaline-extraction of sarcolemmal membranes followed by solubilization with CHAPS, DEAE-Sepharose chromatography, gel filtration HPLC, addition of salt, cholate and lipids and WGA-Sepharose chromatography. Combined in series, these steps provide a 724-fold enrichment in exchange activity over sarcolemma (Table 2) and yield three major proteins of 75, 120 and 140 kDa upon

SDS-PAGE under reducing conditions and one major band centered at 140 kDa under nonreducing conditions (Figure 12). These data are consistent with the recently proposed hypothesis that proteins of 70, 120 and 160 kDa are all involved with Na^+/Ca^{2+} exchange (Philipson *et al.*, 1988; Vemuri *et al.*, 1990; Nicoll*et al.*, 1990) with the exception of minor differences in molecular masses. That is, on our gels, proteins of 75, 120 and 140 kDa correlate with exchange activity. This is further supported by the results obtained using polyclonal antibodies (provided by K. D. Philipson; Philipson *et al.*, 1988) which recognized proteins of 75, 120 and 140 kDa.

We found that exchange activity solubilized with CHAPS readily binds to DEAE-Sepharose in 160 mM NaCl. Selective elution of exchange activity was observed when the ionic strength was increased to 225 mM NaCl. Exchange activity was found to elute at lower ionic strength (180 mM NaCl), but occurred at a slower rate resulting in large elution volumes. These results differ from those of Philipson *et al.* (Philipson *et al.*, 1988) where Na^+/Ca^{2+} exchange activity was reported to elute in parallel with all protein. There are several possible reasons for the discrepancy. In the separation reported here, the solubilized preparation and column equilibration and elution buffers contained 9-12 mM CHAPS (at or above the cmc; Hjelmeland, 1980). A concentration of 3 mM CHAPS resulted in elution of exchange activity in the void volume from the size exclusion columns (data not shown) which is consistent with protein aggregation.

The addition of phospholipids at low levels ("Experimental Procedures") may also contribute to reducing protein-protein interactions and aid protein separation. We found that the inclusion of phospholipids in the equilibration and elution buffers was required for maximal recovery of exchange activity. While omission of phospholipids resulted in recovery of activity below 25% from the ion exchange step, recovery of activity from all fractions approached 100% with the addition of phospholipids. Nicoll and Applebury (1989), working with the ROS Na^+/Ca^{2+} exchanger, also found that the addition of phospholipids was required for the preservation of exchange activity during column chromatography. The absence of phospholipids at the DEAE-chromatography step may account for the high degree of inactivation reported to occur at this step (Philipson et al., 1988). We also observed an inverse relation between recovery of activity and the volume used to wash the column prior to elution at 225 mM NaCl. Loss of activity with increased wash volume may be the result of a gradual leaching of exchange activity off the column (activity can be detected in all wash fractions), inactivation of the exchanger or both.

The addition of phospholipids to the gel filtration HPLC running buffer was also required for maximal recovery of exchange activity. In the absence of phospholipids, recovery of activity fell below 25% while in the presence of phospholipids, recovery of activity from all fractions approached 100%. Gel filtration HPLC of the exchanger-enriched fraction obtained from the

DEAE-Sepharose column provided 561-fold enrichment of exchange activity over sarcolemma.

Although Na^+/Ca^{2+} exchange activity solubilized with CHAPS was quite readily taken up by WGA-Sepharose, little or no selective elution of exchange activity could be achieved with the competing sugar, N-acetyl-D-glucosamine. These results are in agreement with previous reports (Philipson *et al.*, 1988; Durkin *et al.*, 1988). Recently, a less conventional approach, involving the use of nonionic detergents to elute exchange activity from immobilized wheat germ agglutinin, has been reported (Philipson *et al.*, 1988; Durkin *et al.*, 1988). Experiments in this laboratory suggested that WGA-Sepharose binds very little exchange activity in the presence of low cholate, a high concentration of phospholipids and high ionic strength, but retains the ability to bind several sarcolemmal proteins. These conditions were incorporated into the purification scheme and used to remove much of the remaining protein contaminants to yield three proteins of 75, 120 and 140 kDa under reducing conditions and one protein centered at 140 kDa under nonreducing conditions (Figure 12).

The specific activity of our most enriched preparation (3766 nmol/mg/sec) is approximately 3.7- to 5.6–fold higher than previously reported values (Soldati *et al.*, 1985; Philipson *et al.*, 1988; Durkin *et al.*, 1991). This may be due in part to the method of reconstitution. Reconstitution of solubilized sarcolemmal proteins by modification of the procedure of Miyamoto and Racker (1980) as described by

Ambesi *et al.* (1991e) provides a 30–fold enrichment of exchange activity over sarcolemma as compared to the ~5–fold enrichment reported for reconstitution by detergent extraction over Bio–Beads SM–2 (Philipson *et al.*, 1988). However, we must caution that variability exists from laboratory to laboratory in the details of Na^+/Ca^{2+} exchange assays (e.g., concentration of $^{45}Ca^{2+}$ used) and, in particular, the assays used to measure protein in proteoliposome preparations.

A turnover number for the cardiac Na^+/Ca^{2+} exchanger can be determined from initial velocity conditions at 40 μM $[Ca^{2+}]_o$ (approximates Vmax conditions). The specific activity of the purified Na^+/Ca^{2+} exchanger is 3766 nmol/mg/sec (Table 2). If the cardiac Na^+/Ca^{2+} exchanger is a single polypeptide of 140 kDa, then the apparent turnover number at 37°C is 527 $Ca^{2+}s^{-1}$. The actual turnover number is likely to be higher since the exchanger was not purified to homogeneity and some degree of inactivation is expected. There remains some uncertainty as to whether the presence of the 75 or 120 kDa proteins (SDS-PAGE, reducing conditions) reflects the active Na^+/Ca^{2+} exchanger, although chymotrypsin experiments reported by Philipson *et al.* (1988) suggest that they do. Since the 75 and 120 kDa proteins appear to arise from the reduction of disulfide bonds (Philipson *et al.*, 1988; Ambesi *et al.*, 1991c; Figure 12), the question is whether or not the nicked version of the 140 kDa protein retains activity.

If the active Na^+/Ca^{2+} exchanger consists of multiples of the 140 kDa protein (dimer, trimer, etc.), then the turnover number may increase by a factor equal to

the number of monomers per active exchanger. Thus, a functional Na^+/Ca^{2+} exchanger consisting of two 140 kDa proteins (dimer) could have a turnover number of about 1050 Ca^{2+} s^{-1} which is consistent with the estimate of 1000 Ca^{2+} s^{-1} by Cheon and Reeves (1988) using indirect methods. This value, however, falls short of the 2500 Ca^{2+} s^{-1} reported by Niggli and Lederer (1991) using electrophysiological techniques on intact cardiac myocytes. While molecular weights of membrane proteins predicted by size exclusion chromatography must be used with caution (LeMaire *et al.*, 1986), the elution profile in Figure 11 did suggest a large discrepancy in size from that observed with SDS-PAGE (i.e., 370 versus 140 kDa). Hale *et al.* (1988), using cholate solubilized bovine sarcolemma, reported a minimum size of 224 kDa for the exchanger based on size exclusion chromatography. We also observed immunoreactivity in the region of ~250-300 kDa on immunoblots of the purified preparation. These data are consistent with the hypothesis that the functional exchanger can exist as a multimeric complex in the solubilized state. Whether the complex is a homomultimer or heteromultimer remains unknown. More intriguing is the question of whether such complexes exist in the lipid bilayer. In this regard, Hale *et al.* (1988) reported a minimal size of 226 kDa for the exchanger in frozen bovine cardiac sarcolemma based on radiation inactivation-target size analysis.

CHAPTER IV

Na+/Ca2+ Exchanger : Protein and Proteolytic Fragments in Cardiac Sarcolemma

INTRODUCTION

Nicoll *et al.* (1990) recently reported the isolation of a cDNA clone for the canine cardiac Na^+/Ca^{2+} exchanger. The cDNA was characterized by an open reading frame encoding a 108 kDa protein including a potential NH_2-terminal signal sequence and six potential glycosylation sites. Immunoblots of canine sarcolemmal proteins, however, reveal a more complex pattern when probed with polyclonal antibodies raised against purified Na^+/Ca^{2+} exchange preparations (Philipson *et al.*, 1988; Vemuri *et al.*, 1990; Ambesi *et al.*, 1991c) or against a peptide from the deduced amino acid sequence (Nicoll *et al.*, 1990). Under reducing conditions, three proteins of 70-75, 120 and 140-160 kDa manifest immunoreactivity. Under nonreducing conditions, a protein of 140-160 kDa was found to be immunoreactive. Similar profiles have been observed for the purified exchanger by silver stain and immunoblots (Philipson *et al.*, 1988; Ambesi *et al.*, 1991c).

The origin of the three proteins is unclear. They might arise in one of several ways including: a) through differential proteolytic processing of a precursor or parent protein; or b) through proteolysis during tissue processing for the isolation of sarcolemma or microsomal preparations. This paper is focused on these two possibilities. Polyclonal antibodies, raised against the purified cardiac

Na$^+$/Ca^{2+} exchanger, were antigen purified (i.e., from each of the three proteins) and then tested for their ability to immunoprecipitate exchange activity and to cross-react with the other two proteins. In addition, the profile under reducing conditions was compared for isolated membrane preparations and isolated cardiac cells maintained in culture. Use of the latter minimized the time of processing prior to electrophoresis. The results suggested that the 70-75 and 120 kDa proteins are the products of proteolysis that occur during isolation of the membrane fractions.

EXPERIMENTAL PROCEDURES

Materials

Pepstatin A was purchased from Calbiochem, leupeptin from Boehringer Mannheim Biochemicals, PMSF from Serva Biochemicals and CHAPS from Pierce Chemical Corporation. Protein A-Sepharose 6MB was obtained from Pharmacia. All reagents for SDS-PAGE were electrophoresis grade and obtained from Bio-Rad Laboratories. The Centricell 60 filtration device was purchased from Polysciences, Inc. ^{45}CaCl$_2$ was obtained from New England Nuclear. Soybean phosphatidylcholine Type II-S was obtained from Sigma and treated as previously described (Hale *et al.*, 1984). All other chemicals were reagent grade.

Purification of the Na+/Ca2+ Exchanger and Assays

Isolation of sarcolemmal preparations from canine cardiac ventricle, purification and reconstitution of the Na+/Ca2+ exchanger, assays for activity, SDS-PAGE and western blots were carried out as previously described (Ambesi *et al.*, 1991c).

Isolation of Canine Ventricular Myocytes

Canine ventricular myocytes were isolated by collagenase perfusion of a section of the free wall of the left ventricle via a branch of the left circumflex artery according to the procedure of Urabe *et al.* (1991) and were obtained from R. L. Kent. In one experiment, the cells were allowed to settle for 5 min. The suspension above the settled cells (cell debris and round cells) was removed and the procedure was repeated twice. To the settled cells, an equal volume of 2X Laemmli buffer was added (final 2% SDS, 0.5% Triton X–100, 100 mM DTT, 1μM pepstatin, 1 μM leupeptin, 0.1 mM PMSF, 0.1 mM EDTA). The sample was vortexed, heated 3 min at 95-98° and electrophoresed immediately. In a second experiment, the cells were maintained in culture for ~20 hours on laminin–coated culture plates in serum–free Medium 199 (GIBCO BRL) culture media containing penicillin (100 U/ml), streptomycin (0.1 mg/ml) and gentamicin (0.25 mg/ml). Rod shaped cells comprised ~50–80% of the cell population. Culture plates were washed with PBS (3 x 4 ml) and the cells were gently removed with a rubber

policeman into 2 ml of PBS and allowed to settle. The settled cells were treated for SDS-PAGE as above and electrophoresed immediately (~30 min total processing time). Electrophoresed samples were transferred onto Immobilon–P and tested for immunoreactivity (500–fold dilution of antiserum). Immunoreactions were detected by autoradiography using [125I]protein A (Huynh *et al.*, 1985).

Preparation of Microsomal Fractions

Canine kidney and bovine, porcine and rat ventricular tissue was minced, suspended in 5 volumes of 160 mM NaCl, 20 mM Mops–Tris, pH 7.4 and homogenized in a Potter Elvehjem vessel. Homogenates were centrifuged at 8500 x g for 15 min and the pellets discarded. The supernatants were centrifuged (100,000 x g, 60 min) and the pellets (microsomes) were resuspended in 1–2 ml of 160 mM NaCl, 20 mM Mops–Tris, pH 7.4. Microsomes were frozen in liquid nitrogen and stored at -70°.

Immunoprecipitation of Na+/Ca2+ Exchange Activity

Protein A-Sepharose 6MB (0.2 ml) was washed with 4 x 2 ml of MAPS II Binding Buffer (Bio-Rad) and incubated for 30 min at room temperature (gentle agitation) with either 0.5 ml of antiserum or 0.5 ml of preimmune serum diluted with an equal volume of MAPS II Binding Buffer. The protein A-Sepharose beads were washed as above. Sarcolemmal membranes (150 μg) were alkaline-extracted

(Ambesi *et al.*, 1991c), solubilized in 9 mM CHAPS, 160 mM NaCl, 10 mM Mops-Tris, pH 7.4 as previously described (Ambesi *et al.*, 1991e) and incubated with the IgG-protein A-Sepharose complex for 30 min at room temperature with gentle agitation. An aliquot of the supernatant was removed and reconstituted (Ambesi *et al.*, 1991e) and Na^+/Ca^{2+} exchange activity was measured.

Antigen Purification of Antibodies

Sarcolemmal proteins were subjected to preparative SDS-PAGE (290 µg protein added to each of 2 polyacrylamide gels) and transferred onto Immobilon-P. The portions of the blot which contained the molecular weight markers were removed and stained for protein by immersion into 0.1% Naphthol Blue Black (w/v), 45% MeOH (v/v), 10% glacial acetic acid (v/v) for 10 min with rocking. The blot was destained with multiple water washes. Vertical strips (0.5 cm in width; taken adjacent to the molecular weight markers) were cut from the blot and tested for immunoreactivity with antiserum (detection with goat anti–rabbit IgG–horseradish peroxidase conjugate). The remainder of the blot was stained for total protein with Naphthol Blue Black. Subsequently, the blot was realigned and divided into 20 horizontal strips, each representative of a particular antibody binding region or molecular weight range using the immunoblots and Naphthol Blue Black–stained proteins as guides. The strips were blocked in TBS (150 mM NaCl, 20 mM Tris-Cl, pH 7.4) containing 5% nonfat milk and 2% fetal bovine

serum for 1 hour at 37°, washed 3 x 5 min with TBS and incubated in a 500-fold dilution of antiserum in TBS containing 0.05% Tween-20 for either 2 hours at room temperature or 12-18 hours at 5°. Subsequently, the strips were washed as above, separated and the bound antibodies were eluted in 10 ml of 0.5 M NaCl, 0.2 M Glycine-HCl, pH 2.8, 100 µg/ml BSA for 10 min at room temperature. The strips were removed, washed as above, blocked in TBS containing 5% nonfat milk and 2% fetal bovine serum for 15 min at room temperature and the procedure repeated (each repeat = one cycle). The eluted antibodies were adjusted to pH 8.0 by addition of 650 µl of 2 M Tris-base. Antibodies eluted from multiple cycles for each strip were pooled, concentrated to about 500 µl in a Centricell 60 (30,000 Da molecular weight cut-off) and tested for the ability to immunoprecipitate Na^+/Ca^{2+} exchange activity (5 cycles) and react with sarcolemmal proteins on immunoblots (3 cycles).

Protein Assay

Protein was determined by the method of Lowry *et al.* (1951) for sarcolemma and microsomal preparations. Bovine serum albumin was used as the standard.

RESULTS

Immunoprecipitation of Na^+/Ca^{2+} Exchange Activity

Antibodies from antiserum raised against the purified Na^+/Ca^{2+} exchanger

preparation or preimmune serum (Ambesi *et al.*, 1991c) were bound to protein A-Sepharose 6MB and incubated with solubilized, alkaline-extracted sarcolemma as described under "Experimental Procedures". The supernatant was subsequently reconstituted and assayed for Na^+/Ca^{2+} exchange activity. While preimmune serum had little or no effect on recovery of activity, the antiserum immunoprecipitated $97.4 \pm 1.3\%$ (values at 10 min; n = 4) of the Na^+/Ca^{2+} exchange activity when compared to preimmune serum (Figure 14). Furthermore, Na^+/Ca^{2+} exchange activity was immunoprecipitated in a concentration-dependent manner with 50% maximal effect at 20 µl antiserum per mg sarcolemma (Figure 14, *inset*).

Antigen Purification of Antibodies

Sarcolemmal proteins were subjected to SDS-PAGE and transferred to Immobilon-P. Antibodies were bound to and eluted from specific protein regions as described under "Experimental Procedures". The antigen-purified antibodies were tested for their ability to immunoprecipitate Na^+/Ca^{2+} exchange activity (Figure 15) and react with sarcolemmal proteins under reducing and nonreducing conditions (Figure 16). Antibodies eluted from the 75, 120 and 140 kDa proteins immunoprecipitated 92%, 91% and 83% (n = 2), respectively, of the Na^+/Ca^{2+} exchange activity as compared to preimmune serum (Figure 15). In addition, antibodies eluted from the 85 and 114 kDa proteins immunoprecipitated
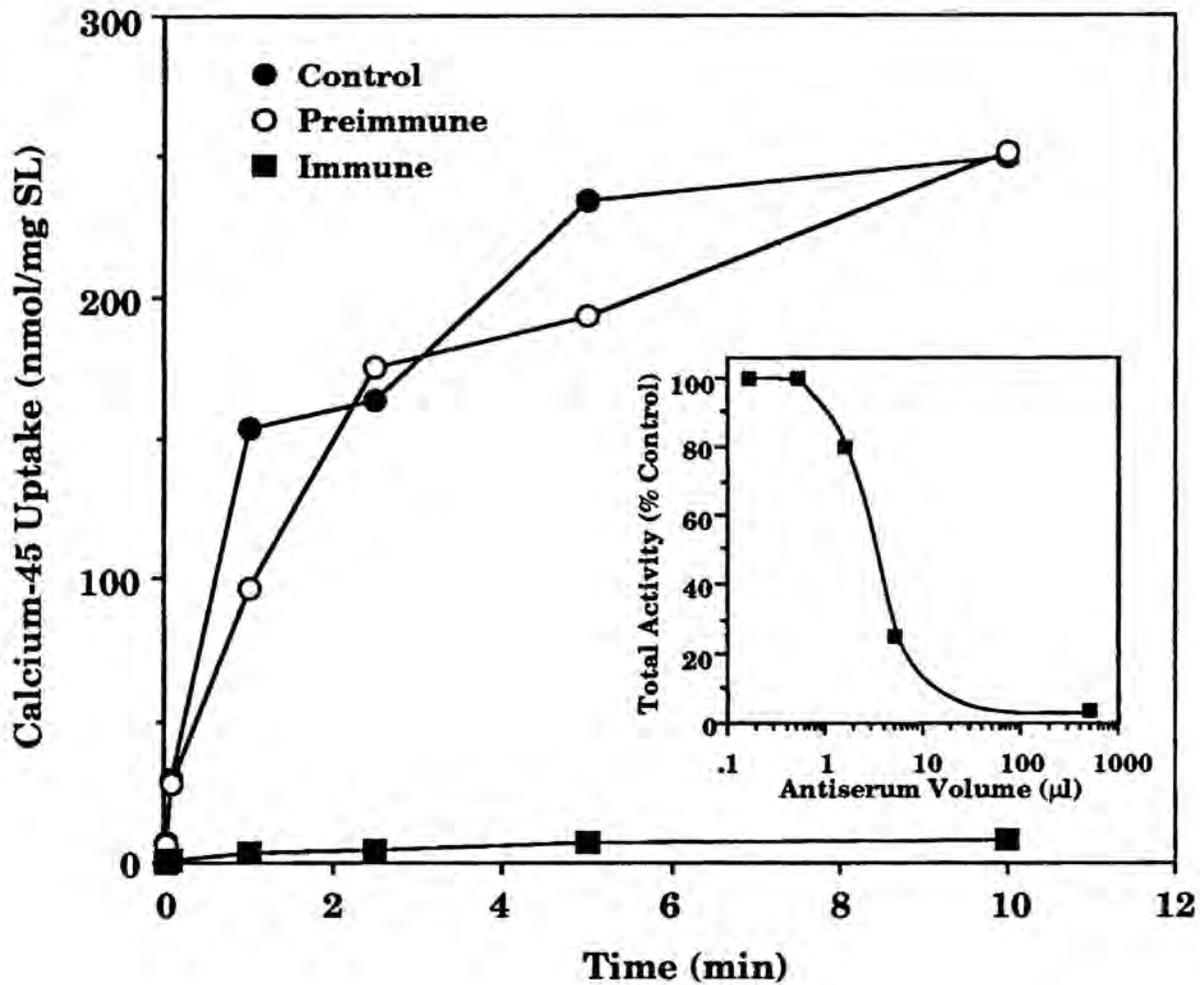
Figure 14. **Immunoprecipitation of Na+/Ca²⁺ Exchange Activity.** Sarcolemmal membranes (150 µg) were alkaline extracted (Ambesi *et al.*, 1991c) and solubilized with 9 mM CHAPS, 160 mM NaCl, 10 mM Mops-Tris, pH 7.4 as previously described (Ambesi *et al.*, 1991e). The solubilized fraction was either reconstituted directly or incubated 30 min with IgG (from preimmune or immune serum) immobilized onto protein A-Sepharose as described under "Experimental Procedures". An aliquot of the supernatant was removed and reconstituted. Calcium-45 uptake into proteoliposomes in the presence of an outwardly directed sodium gradient was determined as a function of assay time at 37° (n=2). *Inset*: Recovery of Na+/Ca²⁺ exchange activity versus volume of antiserum incubated with protein A-Sepharose.
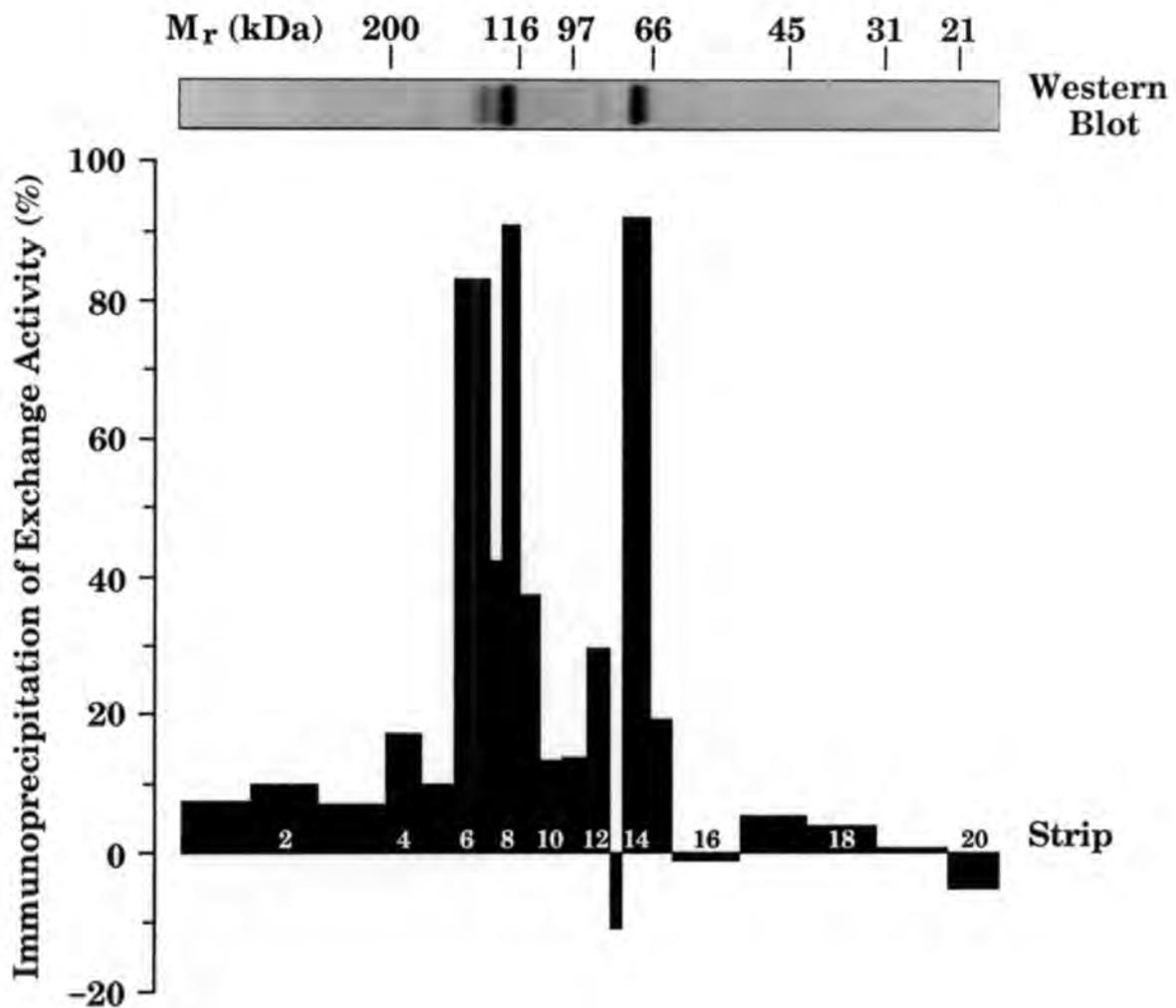
Figure 15. **Antigen Purification of Antibodies: Immunoprecipitation of Na+/Ca2+ Exchange Activity.** Antibodies were antigen purified as described under "Experimental Procedures". Antibodies, eluted from each of the 20 strips (5 cycles), where each strip represents a particular antibody binding region or molecular mass range as indicated by the western blot shown above, were tested for the ability to immunoprecipitate exchange activity. Each bar on the graph represents the degree of immunoprecipitation of Na+/Ca2+ exchange activity achieved with the antibodies eluted from that particular strip.

Figure 16A
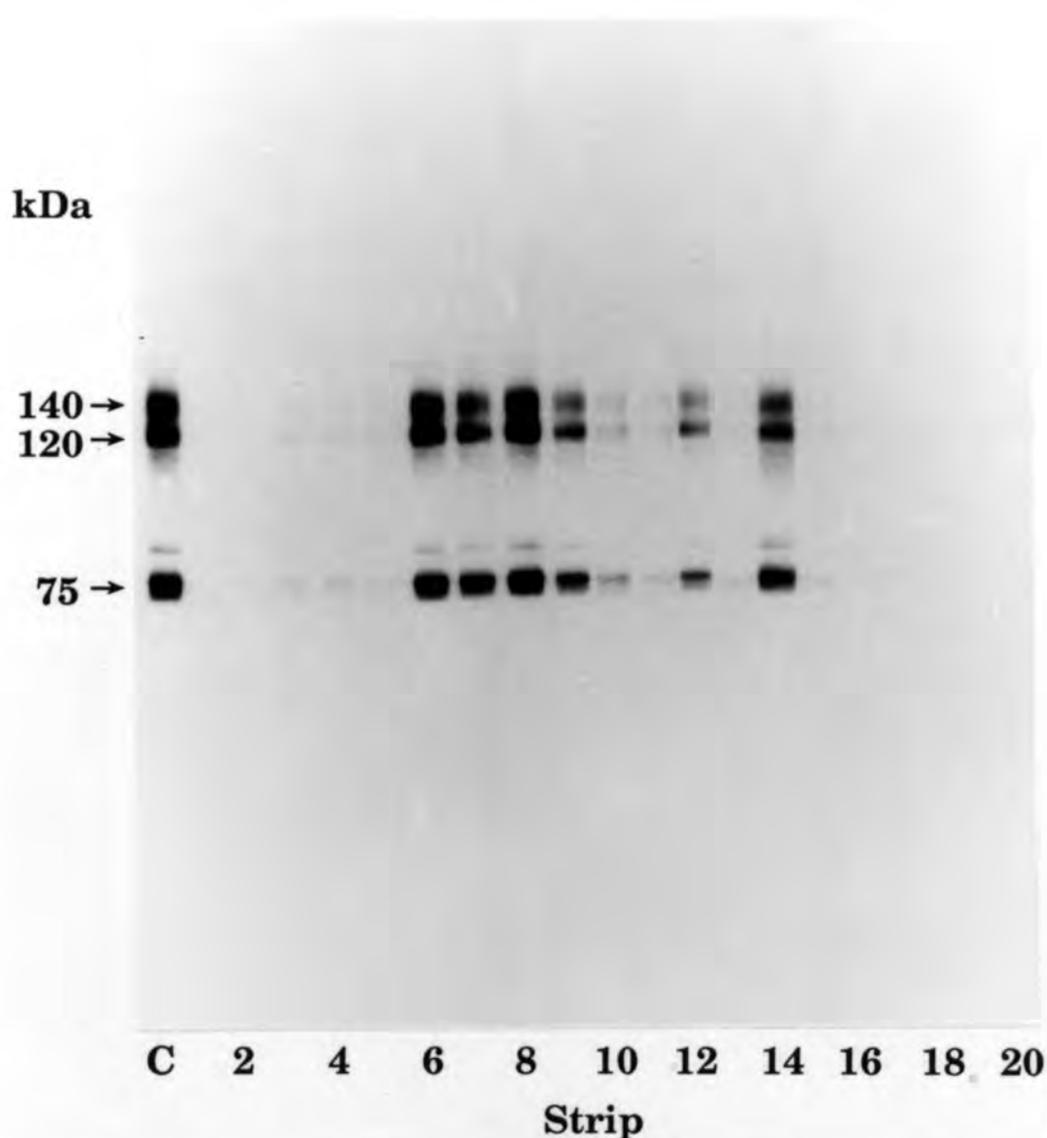
Figure 16. **Antigen Purification of Antibodies: Immunoreactions With Sarcolemmal Proteins.** Antibodies were antigen purified as described under "Experimental Procedures" (3 cycles), diluted into 5 ml of TBS containing 1% BSA, 0.05% Tween-20, and tested for the ability to react with sarcolemmal proteins (10 μg) under reducing (A) and nonreducing (B) conditions. The number below each lane corresponds with the strip from which antibodies were eluted as shown in Figure 15 (e.g., *lanes 6, 8* and *14* represent immunoreactions seen with antibodies eluted from the 140, 120 and 75 kDa proteins, respectively). Control lanes (C) represent immunoreactions of sarcolemmal proteins with whole antiserum (500-fold dilution). Immunoreactions were detected by autoradiography using [125I]protein A.

Figure 16B
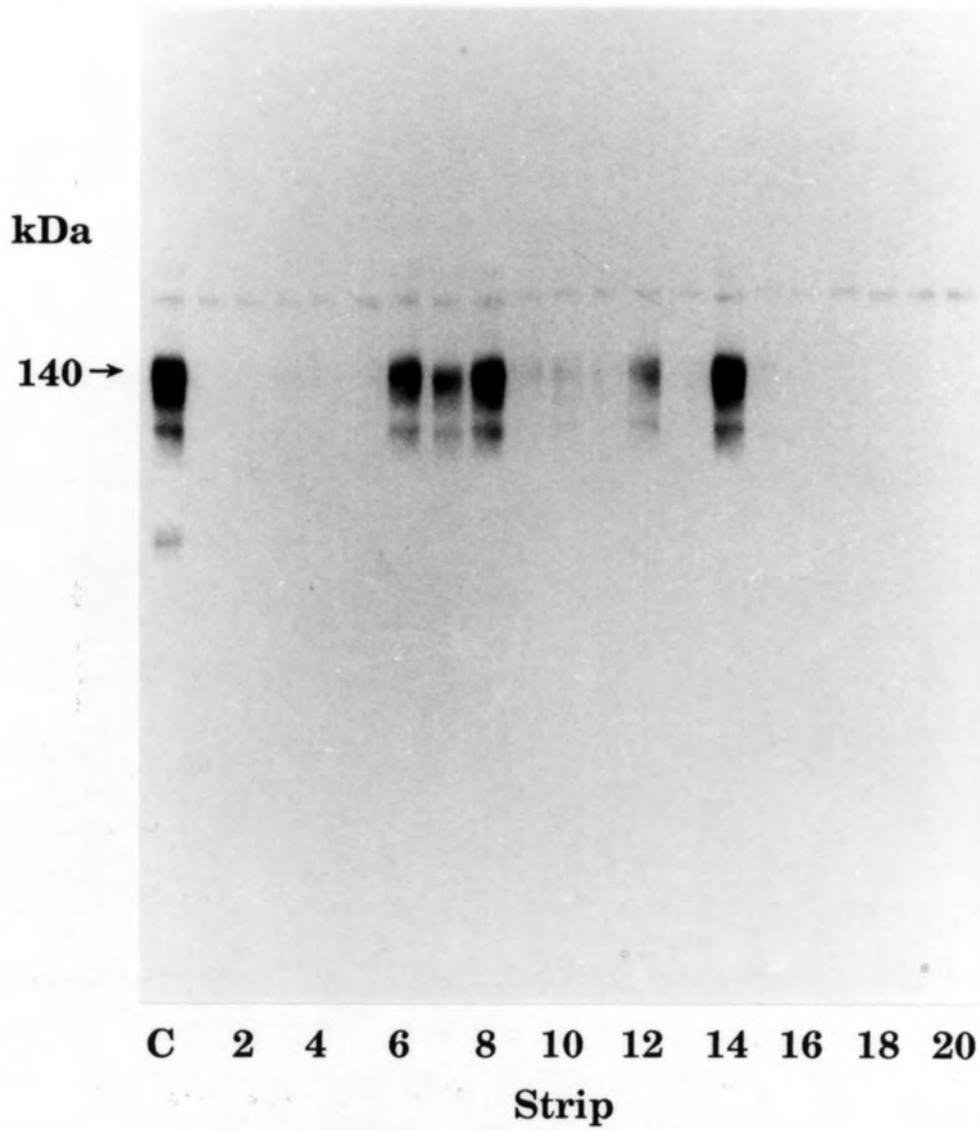
Figure 16. Figure legend on previous page.

30% and 37% (n=2) of the Na^+/Ca^{2+} exchange activity, respectively. Immunoprecipitation with antibodies eluted from nonspecifically bound sites (regions exhibiting no detectable immunoreactivity on immunoblots) averaged 6% (n=2) when compared to preimmune serum.

Antibodies eluted from each of the three major proteins (75, 120 and 140 kDa; strips 14, 8 and 6, respectively) were found to cross-react with each of the other two proteins as well as the minor 85 and 114 kDa proteins under reducing conditions (Figure 16A). Furthermore, antibodies eluted from the 114 and 85 kDa proteins cross-reacted with each of the three major proteins. An identical profile was observed for nonreducing conditions with the strongest reaction seen at 140 kDa (Figure 16B).

Immunoreactions with Isolated Canine Ventricular Myocytes

Polyclonal antibodies generated against the purified cardiac Na^+/Ca^{2+} exchange preparation (Ambesi *et al.*, 1991c) were tested for immunoreactivity against isolated canine ventricular myocytes which were maintained in culture for 20 hours prior to electrophoresis (Figure 17). Under reducing conditions, a very intense reaction with the 140 kDa protein was found. The 120 kDa protein was not detected while only small amounts of the 75 kDa protein (relative to the 140 kDa protein) were observed. Identical results were observed when the cells were used within 60 min of isolation. Thus, it seems unlikely that the difference in profiles
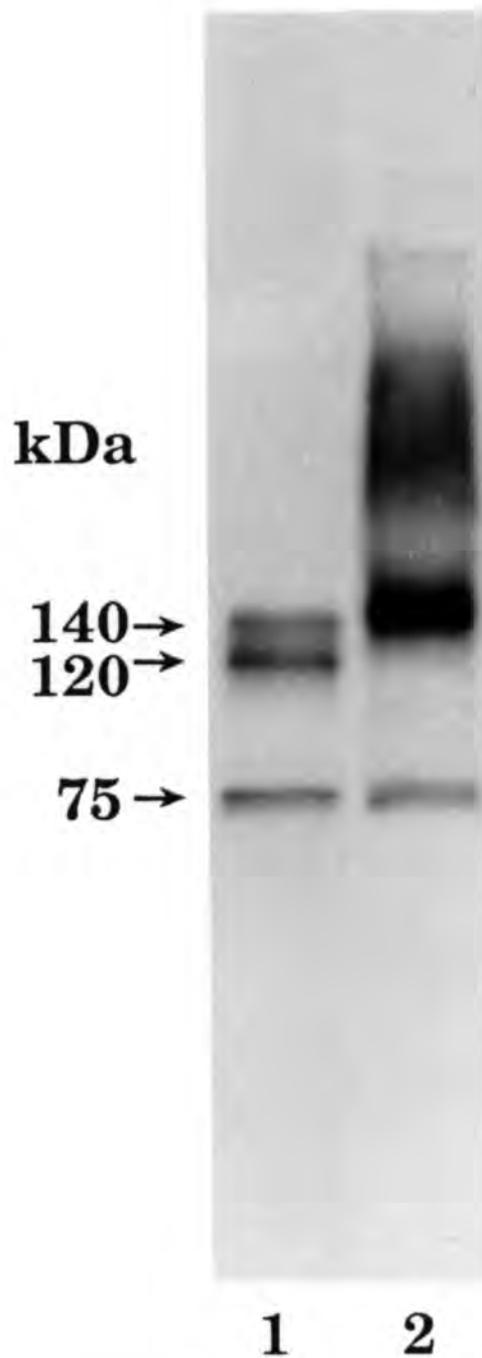
Figure 17. **Immunoreactions With Isolated Canine Ventricular Myocytes.**
Canine ventricular myocytes were isolated as previously described
(Urabe *et al.* , 1991) and maintained in culture for ~20 hours. Culture
plates were rinsed and ventricular myocytes were gently removed with
a rubber policeman into 2 ml of PBS, allowed to settle, solubilized with
electrophoresis sample buffer and subjected to SDS–PAGE
("Experimental Procedures"). Proteins were transferred onto
Immobilon–P and tested for immunoreactivity (500–fold dilution of
antiserum). Immunoreactions were detected by autoradiography
using [125I]protein A. *Lane 1*, 200 ng canine cardiac sarcolemma; *lane
2*, ~7500–12,500 ventricular myocytes.

for isolated membranes and isolated cells reflects the quiescent state of the cultured cells prior to SDS-PAGE.

Immunoreactions with Canine Kidney and Bovine, Porcine and Rat Heart and Microsomes

In bovine, porcine and rat heart microsomes, the polyclonal antibodies generated against the purified canine cardiac Na^+/Ca^{2+} exchange preparation (Ambesi *et al.*, 1991c) were found to react with proteins that appeared to be comparable to the 120 and 140 kDa proteins seen in canine cardiac sarcolemma (Figure 18). Although proteins of comparable molecular weight were detected in canine kidney microsomes, immunoreactivity with these proteins was greatly reduced. Greatest variability in the immunoreactive patterns among the microsomal fractions occurred in the 75 kDa region. While bovine and porcine heart microsomes exhibited reactivity in the 75 and 85 kDa regions, canine kidney and rat heart microsomes did not. In addition, there were variations among the microsomal fractions in the number, relative amounts and molecular weights of the minor immunoreactive proteins detected.

DISCUSSION

When SDS-PAGE is carried out under reducing conditions, the canine
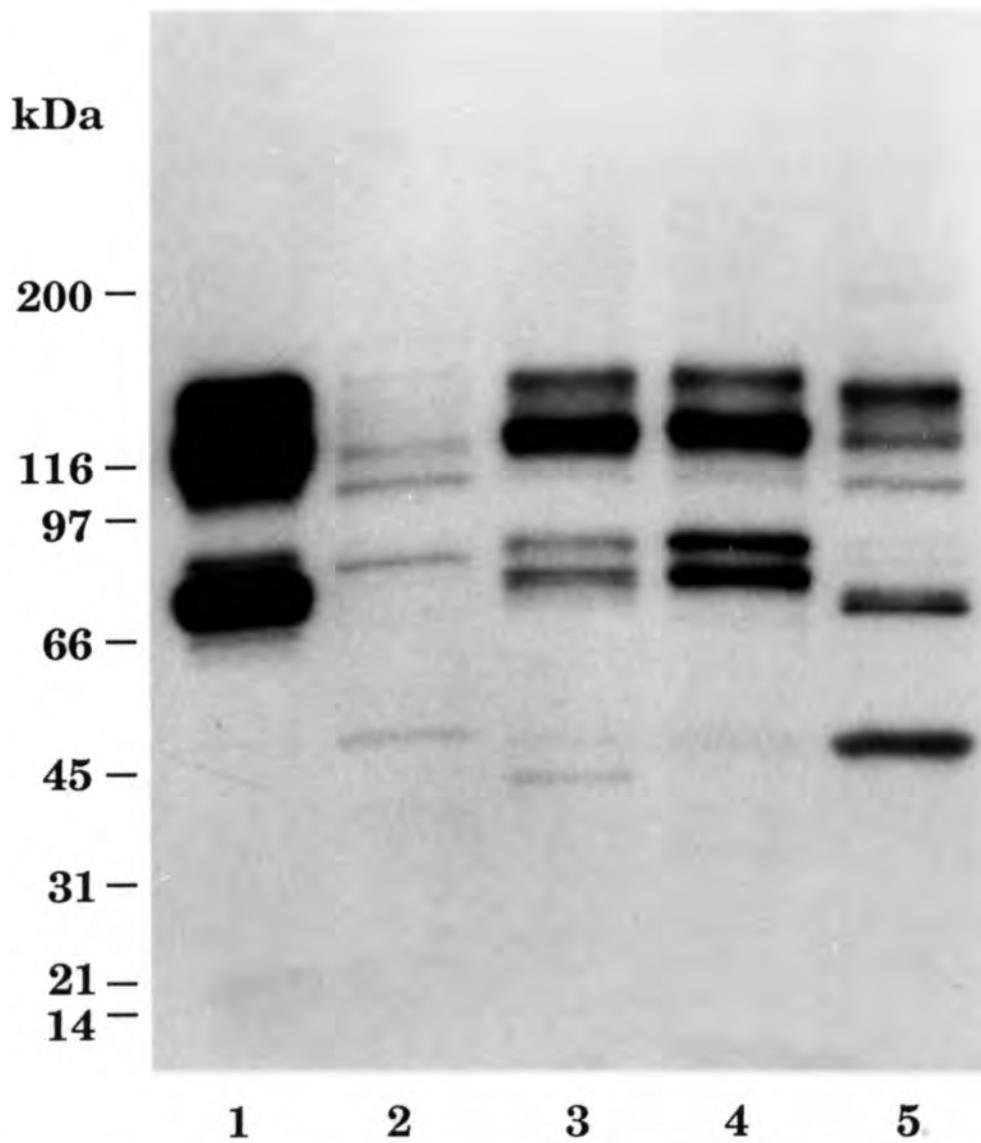
Figure 18. **Western Blot of Canine Cardiac Sarcolemma and Canine Kidney and Bovine, Porcine and Rat Heart Microsomes.** Microsomal fractions were prepared as described under "Experimental Procedures", subjected to reducing SDS–PAGE and transferred onto Immobilon–P. Western analysis was carried out using a 500-fold dilution of antiserum and immunoreactions were detected by autoradiography using [125I]protein A. Molecular weight markers are shown on the left. *Lane 1*, 0.5 µg canine cardiac sarcolemma; *lane 2*, 10 µg canine kidney microsomes; *lane 3*, 10 µg bovine heart microsomes; *lane 4*, 10 µg porcine heart microsomes; *lane 5*, 10 µg rat heart microsomes.

cardiac Na^+/Ca^{2+} exchanger is characterized by three protein bands that are related to each other. Philipson *et al.* (1988) first reported that the 70 and 120 kDa proteins share common epitopes for immunoblotting by use of antigen-purified antibodies. Subsequently, Nicoll *et al.* (1990) reported that antibodies generated against a synthetic peptide constructed from the deduced amino acid sequence (amino acids 680-694), recognize the three proteins on immunoblots. This suggests that all three bands contain this segment of amino acids which is located in the COOH-terminus half of the deduced sequence (i.e., total length of 970 amino acids; Nicoll *et al.*, 1990). Durkin *et al.* (1991) recently reported that the NH_2-terminal sequences of comparable 120 and 160 kDa proteins from bovine cardiac sarcolemma are identical. As shown above, antibodies, antigen-purified from each of the three major proteins, were found to cross-react with each of the other two prominent proteins on immunoblots, as well as the minor 85 and 114 kDa proteins (Figure 16), and to immunoprecipitated 83-92% of the exchange activity (Figure 15). Thus, the three proteins not only contain common epitopes for immunoblotting but also contain common epitopes necessary for immunoprecipitation of exchange activity.

A straightforward interpretation of the profiles observed for reducing and nonreducing conditions is that much of the exchanger protein is nicked by proteolysis but is held together in the sarcolemma (and upon solubilization under nondenaturing conditions) by disulfide bonds and hydrophobic interactions. With

regard to this possibility, Philipson *et al.* (1988) showed that the 70 kDa protein could result from proteolysis of the 120 kDa protein by treatment with chymotrypsin. On the other hand, they were unable to alter the profile seen under reducing conditions by the inclusion of PMSF in the media used for purification. Likewise, we found that the pattern of three proteins was not altered by the inclusion of pepstatin, leupeptin and PMSF in media used for isolation of sarcolemma and purification of the Na^+/Ca^{2+} exchanger (data not shown). Nonetheless, one or more enzymes unaffected by these inhibitors could have caused the modifications.

The isolation of sarcolemma preparations in this laboratory takes approximately eight hours. The isolation of microsomal preparations, which yielded similar profiles on SDS-PAGE under reducing conditions (Figure 18), took approximately two hours. In an attempt to reduce the chance for proteolytic breakdown during processing, and to minimize contamination by other cell types (e.g., endothelial cells and nerve terminals), an experiment was carried out using isolated canine ventricular myocytes maintained in culture. Total elapsed time between removal of the cells from culture plates and application to the gel was ~30 min. Immunoblots of the cells revealed a profile that is clearly different from that for isolated membrane preparations (Figure 17). This profile suggested the following conclusion: The 140-160 kDa protein is the parent protein of the cardiac Na^+/Ca^{2+} exchanger; much of this protein is nicked by proteolytic enzymes during

processing for membrane preparations which gives rise to the 70-75 kDa and 120 kDa proteins and, possibly, to other fragments observed under reducing SDS-PAGE. The results do not eliminate the possibility that some nicking occurs *in vivo* since a small amount of the 75 kDa protein was always present. However, the 75 kDa protein could have been produced during the 30 min processing or could reflect the presence of some nonviable cells on the culture plate.

Durkin *et al.* (1991) suggested that the 160 kDa protein from bovine cardiac sarcolemma could be an artifact of sample preparation for SDS-PAGE. They observed that the intensity of the 160 kDa protein increased while the intensity of the 120 kDa protein decreased when the samples were boiled. We compared heating at 37° for 30 min to heating at 95-98° for three min (the latter routinely used; "Experimental Procedures") for sample preparation. Heating at 37° eliminated the 140 kDa protein, reduced the 120 kDa protein to trace amounts, was associated with the appearance of a 114 kDa protein as the major band, and converted the 75 kDa protein to a 70 kDa protein (data not shown). This downward shift in molecular weights was in the direction expected for proteolysis since heating at 95-98° should favor denaturation of most proteolytic enzymes.

The 70-75 and 120 kDa fragments are, therefore, concluded to be present in the 140-160 kDa protein that is observed under nonreducing conditions. The 70-75 kDa band could include both halves of the 140-160 kDa parent protein due to a nick in the middle of the protein. On the other hand, the remainder of the parent

protein that should be associated with the 120 kDa fragment (20-40 kDa) was not observed under reducing conditions. Presumably, the missing fragment was present in the preparation since the parent protein, but not the 120 kDa protein, was observed under nonreducing conditions. Thus, under reducing conditions, the missing fragment could have been lost due to aggregation (failure to penetrate the resolving gel) or to multiple nicks (ran off the resolving gel). Alternatively, the antibodies may not have recognized this fragment on immunoblots.

There is evidence that the nicked protein manifests exchange activity. Philipson *et al.* (1988) treated purified Na^+/Ca^{2+} exchange preparations with chymotrypsin to the point that severe downward shifts in molecular weights of the proteins were observed without changes in Na^+/Ca^{2+} exchange activity. Similarly, we have noted variations in the relative intensities of the 75, 120 and 140 kDa proteins on immunoblots of sarcolemma preparations that have no obvious correlation with the Na^+/Ca^{2+} exchange activity of these preparations. The relative exchange activities of the nicked versus the intact Na^+/Ca^{2+} exchanger protein, however, remain unknown.

The major implication of this study is that the active Na^+/Ca^{2+} exchanger in cardiac tissue probably exists as a monomer or, possibly, multimer (Ambesi *et al.*, 1991c) of an intact, non-nicked protein. If so, the activation of exchange activity by treatment with proteinases seen *in vitro* (Philipson and Nishimoto, 1982b) may not reflect a means by which the exchanger is regulated *in vivo*.

CHAPTER V

Effect of Polyclonal Antibodies
on the Cardiac Sodium/Calcium Exchanger

We have previously reported the purification of the cardiac sodium/calcium exchanger from canine myocardium and the generation of polyclonal antibodies against the purified exchanger (see chapter III). The polyclonal antibodies immunoprecipitated 97% of the sodium/calcium exchange activity from detergent-solubilized sarcolemma (Figure 14) and reacted with prominent proteins of 75, 120, and 140 kDa (reducing conditions; Figure 12) on immunoblots of the purified exchanger. Only one major protein, centered at 140 kDa, was detected under nonreducing conditions. Subsequently, antibodies against the 75, 120, and 140 kDa proteins were antigen-purified and found to immunoprecipitate 92, 91, and 83% of the exchange activity, respectively (Figure 15). Furthermore, the antigen-purified antibodies exhibited cross-reactivity with each of the other two prominent proteins (Figure 16). These data are consistent with those reported by Philipson *et al.* (1988), with the exception of minor differences in the molecular weights reported, and suggest that the three proteins are immunologically related and that all are related to the cardiac sodium/calcium exchanger.

The purpose of the present study was to determine the effect of these antibodies on sodium/calcium exchange activity manifested by sarcolemmal vesicles (70% sealed R/O; 1-12% sealed I/O; remainder leaky) from canine ventricle. The vesicles were exposed to increasing concentrations of affinity-purified IgG from preimmune or immune serum. Antibodies from the immune

serum stimulated exchange activity 3.5-fold in a dose-dependent manner with half-maximal stimulation at 0.5 µg IgG/µg sarcolemmal protein (Figure 19). Conversely, IgG from preimmune serum had little or no effect between 0.01 and 10 µg IgG/µg sarcolemmal protein. A separate experiment was carried out to test further whether the stimulation observed with IgG from immune serum (anti-NCX) was specific (Figure 20). Four IgG fractions were tested: (1) from the rabbit that was subsequently immunized against the sodium/calcium exchanger (preimmune); (2) anti-NCX; (3) from a rabbit immunized against total sarcolemmal proteins (anti-SL); and (4) from a rabbit immunized against a prominent sarcolemmal protein of 82 kDa enriched by a protease treatment (anti-82; Hale *et al.*, 1984). Exposure to 3 µg IgG/µg sarcolemmal protein showed that the preimmune and the anti-82 fractions caused an increase in sodium/calcium exchange activity of 50 and 46%, respectively, whereas the anti-NCX increased activity by 184% (*inset*, Figure 19). Interestingly, the anti-SL caused nearly as much of an increase (145%) as the anti-NCX but neither the anti-SL nor anti-82 sera were able to immunoprecipitate sodium/calcium exchange activity from solubilized sarcolemma. Eadie-Hofstee plots suggested that the stimulation by anti-NCX and anti-SL was due to a small increase in V_{max} and a larger decrease in the $K_{0.5}$ for $[Ca^{2+}]$ (Figure 20).

Clearly these IgG fractions are capable of causing some nonspecific stimulation of sodium/calcium exchange activity (results with preimmune and
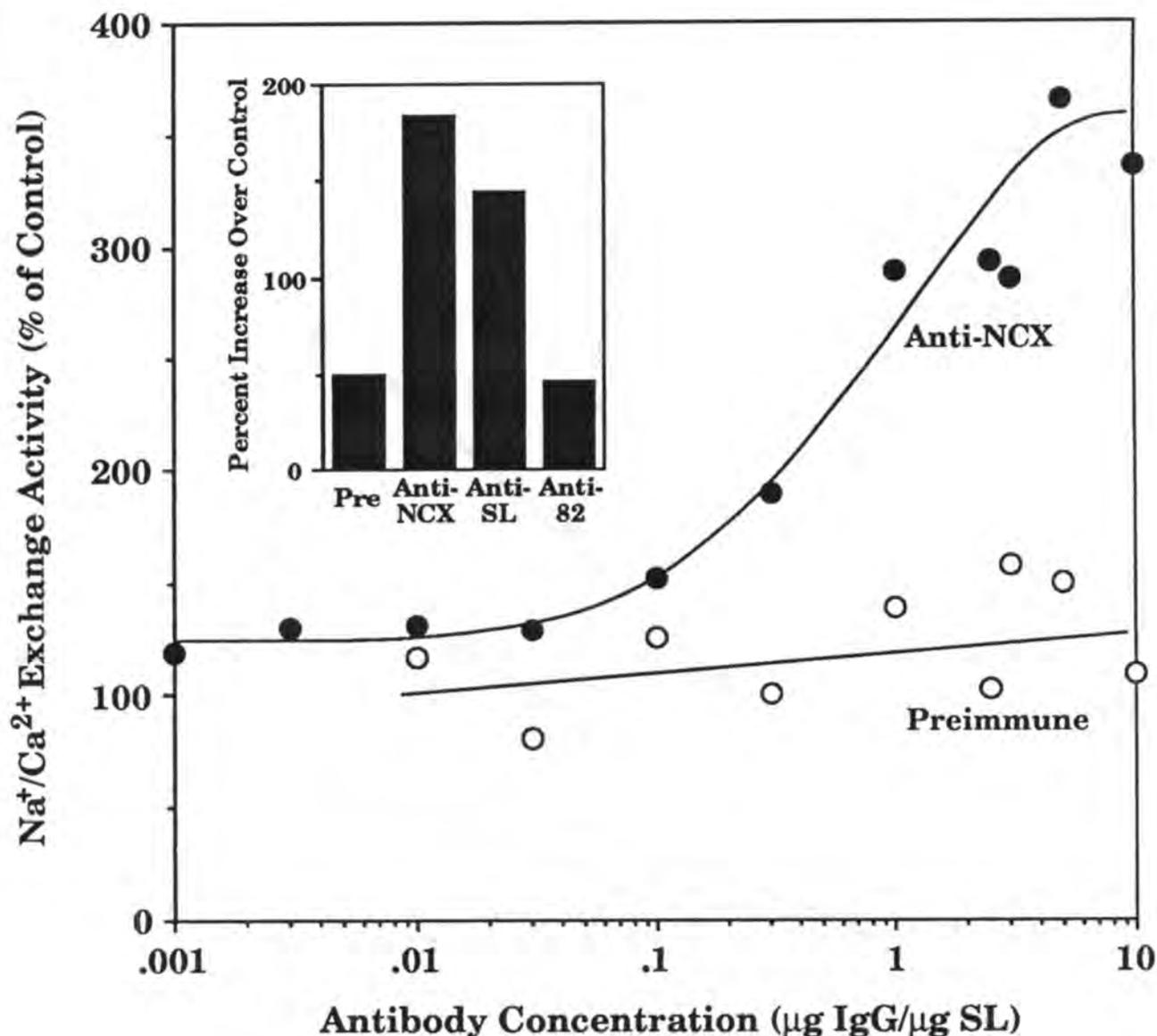
Figure 19. **Effect of Polyclonal Antibodies on Sodium/Calcium Exchange Activity.** Sodium-loaded (160 mM NaCl, 10 mM Mops-Tris, pH 7.4) sarcolemmal vesicles were exposed for one hour at 37° to varying concentrations of affinity-purified IgG from preimmune or immune (anti-NCX) serum and assayed for exchange activity (n = 1-4). *Inset*: Sarcolemmal vesicles were exposed to affinity-purified IgG (3 µg IgG/µg sarcolemmal protein) from preimmune serum, anti-NCX, anti-SL or anti-82 and then assayed for exchange activity (n = 4-5). Sodium/calcium exchange assays were carried out using 40 µM outside [45]Ca/CaCl₂.
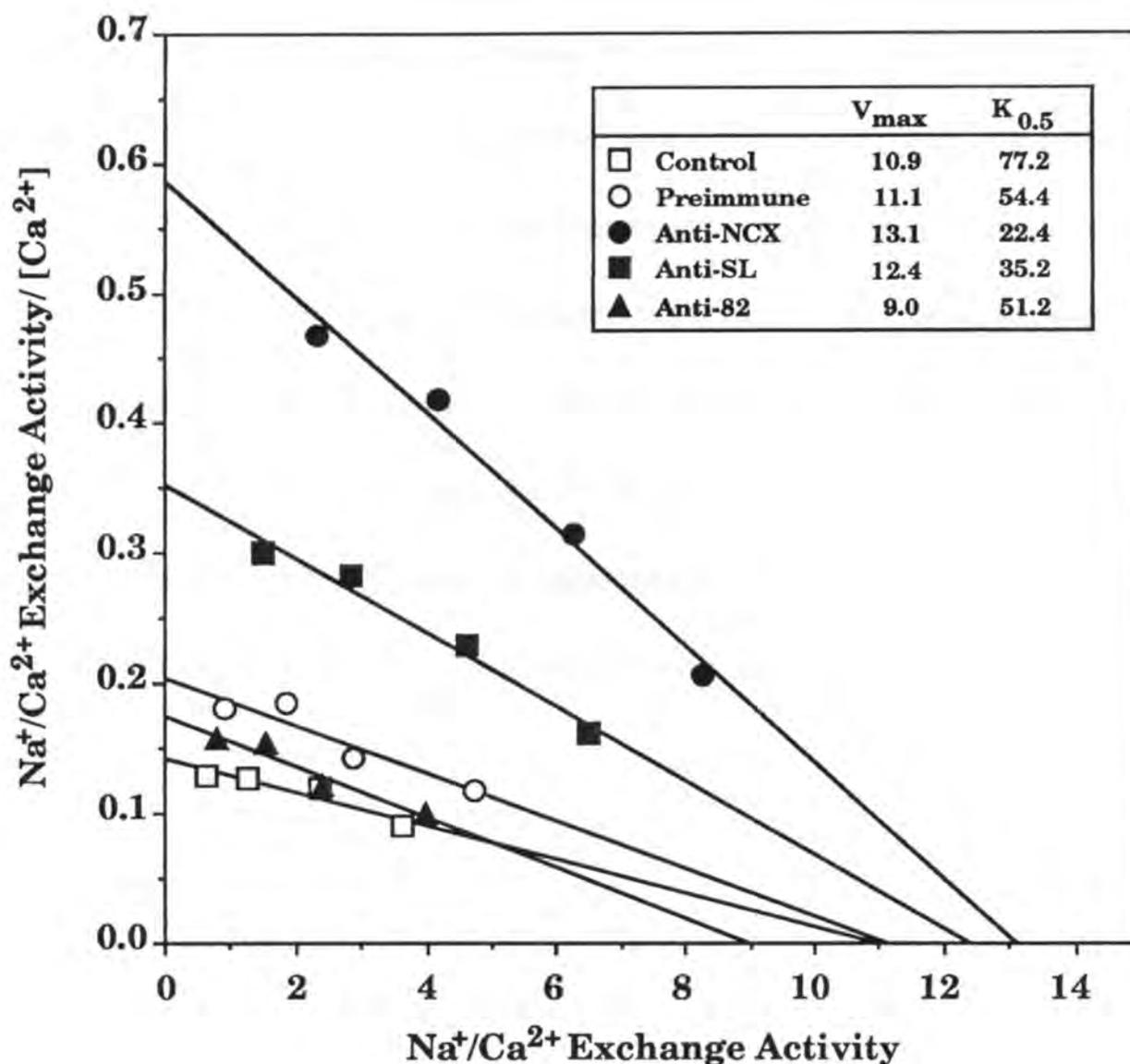
Figure 20. **Effect of Polyclonal Antibodies on Sodium/Calcium Exchange Activity Versus Outside Calcium Concentration.** Sodium-loaded sarcolemmal vesicles were exposed to affinity-purified IgG at 3 µg IgG/µg sarcolemmal protein for one hour at 37°. Sodium/calcium exchange activity was then assessed at varying $[Ca^{2+}]_o$ (5-40 µM). Results (n = 2) are plotted as $v/[Ca^{2+}]_o$ versus v. Least squares analysis was used to determine values (*inset*) for V_{max} (nmol/mg/sec) and $K_{0.5}$ (µM).

anti-82 fractions). The cause of the larger stimulation seen with the anti-NCX and anti-SL fractions is uncertain. Several possibilities are: (1) stimulation by both the anti-NCX and anti-SL reflect nonspecific interactions; (2) stimulation may reflect interaction of the anti-NCX with the exchanger protein and of the anti-SL with a closely associated protein in the sarcolemmal membrane; or (3) the effects reflect interaction of the anti-NCX and a subfraction of the anti-SL with the exchanger protein since inability of the anti-SL to immunoprecipitate the exchanger in one form (detergent-solubilized) does not necessarily preclude specific interactions of a subset of antibodies in the IgG fraction with the exchanger in another form (sarcolemmal membrane).

CHAPTER VI

General Discussion and Future Directions

In the following section, several lines of evidence which support the conclusive identification of the cardiac Na+/Ca²⁺ exchanger as a protein of 140-160 kDa are presented and discussed.

The remaining sections will focus on Na+/Ca2+ exchange in other tissues, how the antibodies developed against the cardiac Na+/Ca2+ exchanger in this laboratory are being used elsewhere and a brief discussion of questions remaining to be answered and general future studies.

IDENTIFICATION OF THE CARDIAC Na+/Ca²⁺ EXCHANGER

Since Hale *et al.* (1984) first reported the correlation of an 82 kDa protein from bovine sarcolemma with Na+/Ca2+ exchange activity, a number of proteins ranging in molecular mass from 33 kDa to as large as 220 kDa have been proposed to be involved with Na+/Ca2+ exchange (Barzilai *et al.*, 1984; Soldati *et al.*, 1985; Longoni *et al.*, 1987; Cook and Kaupp, 1988; Hale *et al.*, 1988; Philipson *et al.*, 1988; Nicoll and Applebury, 1989; Durkin *et al.*, 1990; Ambesi *et al.*, 1990). Identification has typically been based on the correlation of a specific band detected on SDS-polyacrylamide gels with exchange activity. Although a number of minor bands were often detected, they were, for the most part, rejected as possible candidates. As this project was started, Philipson *et al.* (1988) reported purification of three

proteins (70, 120 and 160 kDa) from canine ventricle which appeared to correlate with exchange activity. They pointed out, however, that a number of minor bands were also detected. Their most convincing evidence was based on polyclonal antibodies generated against the enriched preparation. The antibodies immunoprecipitated 96% of the exchange activity from detergent-solubilized sarcolemma and recognized the three major proteins on immunoblots. In addition, a number of minor bands were detected. Curiously, prolonged exposure to chymotrypsin resulted in loss of signal in the three bands on immunoblots while exchange activity was unaffected. Whether one or more of the three bands were involved with Na^+/Ca^{2+} exchange across cardiac sarcolemma was not conclusively determined at that time. Thus, the proteins proposed by Philipson et $al.$ (1988) fell within the same category as those previously reported - inconclusive. Moreover, data concerning the identification of the retinal rod outer segment Na^+/Ca^{2+} exchanger conflicted with those obtained on the cardiac exchanger (based on apparent molecular weights observed on SDS-polyacrylamide gels and antibody cross-reactivity studies). At that time, the two exchangers were presumed to be identical proteins. Indeed, this was a confusing time for all in pursuit of the identification of the Na^+/Ca^{2+} exchanger.

The protein responsible for Na^+/Ca^{2+} exchange across cardiac sarcolemma has now been conclusively identified as a protein of 140-160 kDa. Evidence supporting this conclusion is based primarily upon data obtained from:

(1) Purification studies (Philipson *et al.*, 1988; Ambesi *et al.*, 1991a; Durkin *et al.*, 1991; chapter III);

(2) polyclonal antibodies against the canine cardiac Na^+/Ca^{2+} exchanger developed by two independent laboratories (Philipson *et al.*, 1988; Ambesi *et al.*, 1991a; Ambesi *et al.*, 1991b; Ambesi *et al.*, 1991c);

(3) molecular cloning of the canine cardiac sarcolemmal Na^+/Ca^{2+} exchanger (Nicoll *et al.*, 1990).

Philipson *et al.* (1988) reported purification of the Na^+/Ca^{2+} exchanger from canine ventricle and correlated proteins of 70 and 120 kDa (reducing conditions) with a relatively high specific activity (654 nmol/mg/sec). Under nonreducing conditions, a single protein was detected with a molecular mass of 160 kDa. Subsequently, Ambesi *et al.* (1990) and Durkin *et al.* (1990) reported purification of the Na^+/Ca^{2+} exchanger from canine and bovine cardiac sarcolemma, respectively, and correlated proteins of 70 and 120 kDa (Ambesi *et al.*, 1990) and 150 kDa (Durkin *et al.*, 1990) with specific activities of 2791 nmol/mg/sec (Ambesi *et al.*, 1990) and ~1 µmol/mg/sec (Durkin *et al.*, 1990). Later, proteins of 75, 120 and 140 kDa (specific activity = 3766 nmol/mg/sec; Ambesi *et al.*, 1991a) and 120 and 160 kDa (specific activity = 1µmol/mg/sec; Durkin *et al.*, 1991) were reported to be associated with exchange activity. Thus, data obtained by three independent

laboratories agreed quite closely with respect to the proteins observed in their purified preparations.

Polyclonal antibodies generated by Philipson *et al.* (1988) against the partially purified preparation immunoprecipitated 96% of the exchange activity form detergent-solubilized sarcolemma and recognized proteins of 70, 120 and 160 kDa on immunoblots of sarcolemmal proteins. These antibodies were kindly provided to this laboratory by K. D. Philipson and were found to immunoreact with proteins of 75, 120 and 140 kDa (reducing conditions) in our purified Na^+/Ca^{2+} exchange preparation as well as in our sarcolemma preparations (Ambesi *et al.*, 1991c). Moreover, these antibodies also recognized proteins of 70, 120 and 160 kDa purified from bovine heart (identified as the cardiac Na^+/Ca^{2+} exchanger; Durkin *et al.*, 1991).

The polyclonal antibodies generated against the purified Na^+/Ca^{2+} exchange preparation developed in this laboratory (Ambesi *et al.*, 1991a; chapter III and IV) immunoprecipitated 97% of the Na^+/Ca^{2+} exchange activity from detergent-solubilized sarcolemma and immunoreacted with proteins of 75, 120 and 140 kDa (reducing conditions) in our purified Na^+/Ca^{2+} exchange preparation and sarcolemma preparations. Furthermore, the antibodies directed against each of the three proteins (75, 120 and 140 kDa) were antigen-purified and shown to immunoprecipitate 92, 91 and 83%, respectively, of the exchange activity from detergent-solubilized sarcolemma (chapter IV). The antigen-purified antibodies

also cross-reacted with each of the other two proteins on immunoblots of sarcolemmal protein (chapter IV). These data suggest that the three proteins observed in this laboratory (75, 120 and 140 kDa) are all associated with Na^+/Ca^{2+} exchange across cardiac sarcolemma and appear to be identical to those observed by Philipson and associates (Philipson *et al.*, 1988; Vemuri *et al.*, 1990; Nicoll *et al.*, 1990) and by Reeves and associates (Durkin *et al.*, 1990; Durkin *et al.*, 1991).

Evidence obtained from proteolytic treatment of the purified Na^+/Ca^{2+} exchanger (Philipson *et al.*, 1988), amino acid sequencing of the NH_2-terminal regions of the 120 and 160 kDa proteins (Durkin *et al.*, 1991) and immunoblots of isolated canine ventricular myocytes (chapter IV) also support the hypothesis that the 75 and 120 kDa proteins are proteolytic fragments of the 140 kDa protein.

The polyclonal antibodies generated against the purified canine cardiac exchanger preparation (Philipson *et al.* , 1988) were subsequently used to isolate a cDNA clone leading to expression of functional Na^+/Ca^{2+} exchange activity in *Xenopus* oocytes (Nicoll *et al.*, 1990). The cDNA codes for a protein of 907 amino acids (~108 kDa) possessing six potential glycosylation sites and a single potential phosphorylation site. Antibodies developed against a synthetic peptide from the deduced amino acid sequence immunoreact with sarcolemmal proteins of 70, 120 and 160 kDa on immunoblots which supports the earlier results of Philipson and associates (Philipson *et al.* , 1988; Vemuri *et al.* , 1990) as well as those reported in chapters III and IV. The cardiac exchanger possesses a 32 amino acid signal

sequence as determined by NH_2-terminal amino acid sequencing of the 120 and 160 kDa proteins (amino terminals for the 120 and 160 kDa proteins from bovine heart are identical to one another and to those from canine heart) extracted from SDS-polyacrylamide gels (Durkin *et al.*, 1991).

There is also a consensus sequence for a calmodulin binding site. This was further tested using synthetic peptides. A peptide based on the deduced amino acid sequence corresponding to the potential calmodulin binding site was synthesized and tested for the ability to bind calmodulin (Li *et al.*, 1991). The peptide did, indeed, bind calmodulin with high affinity and was also shown to inhibit exchange activity in sarcolemmal vesicles as well as in intact ventricular cells and giant excised membrane patches (the peptide has been termed 'XIP' for exchanger inhibitory peptide).

Several explanations could account for the discrepancy between the molecular mass observed on SDS-PAGE (140-160 kDa) and that from the deduced amino acid sequence (108 kDa minus a 32 amino acid signal sequence; Durkin *et al.*, 1991). Firstly, the apparent molecular weight may reflect anomalous behavior noted for many proteins upon SDS-PAGE in which proteins migrate differently from their actual molecular weight (Andrews, 1986). This may be due to incomplete SDS binding (due to protein glycosylation or incomplete protein unfolding; Andrews, 1986). Secondly, the molecular weight deduced from the cDNA does not account for glycosylation. Given that there are six potential

glycosylation sites (Nicoll *et al.* , 1990), the exchanger may be heavily glycosylated. Furthermore, the difference may reflect a combination of glycosylation and anomalous behavior upon SDS-PAGE.

Radiation inactivation studies (Hale *et al.*, 1988; Smith and Lindenmayer, in preparation) and results from size exclusion chromatography (Hale *et al.*, 1988; chapter III) suggest that the molecular weight of the *functional* complex responsible for Na^+/Ca^{2+} exchange is much larger than that observed on SDS-PAGE or obtained from the deduced amino acid sequence. Thus, the active exchanger may be multimeric. Protein regulators (inhibitors and/or activators of exchange activity) that might contribute to a multimeric complex, however, have not as yet been observed.

Na^+/Ca^{2+} EXCHANGE IN OTHER TISSUES

In addition to cardiac tissue, Na^+/Ca^{2+} exchange activity has been observed in neuronal tissue (Schellenberg and Swanson, 1981; Gill *et al.*, 1981; Coutinho *et al.*, 1983; Barzilai *et al.*, 1984; Fontana and Blaustein, 1991), renal proximal tubules (Lorenzen *et al.*, 1985; Dominguez *et al.*, 1991), epithelial cells (Taylor, 1989), skeletal muscle (Hidalgo, 1991), smooth muscle (Blaustein, 1989), neutrophils (Dale and Simchowitz, 1991), red blood cells (Milanick and Frame, 1991), retinal rod cells (Lagnado and McNaughton, 1989), platelets (Haynes *et al.* ,

1991) and endothelial cells (Hansen and O'Donnell, 1991).

Solubilization and reconstitution of functional Na+/Ca2+ exchange activity from plasma membranes isolated from rat brain synaptic plasma membranes (Barzilai *et al.*, 1984), aortic smooth muscle (Matlib and Reeves, 1987) and retinal rod outer segments (Cook and Kaupp, 1988; Nicoll and Applebury, 1989) have been reported. However, activities are consistently lower than those observed from cardiac tissue. Matlib and Reeves (1987) solubilized plasma membrane vesicles from rat mesenteric arteries with cholate/asolectin and reconstituted by detergent dilution. Activities in reconstituted vesicles were enriched 4-fold over isolated native membranes (native membranes = 0.5 nmols/mg/sec; reconstituted vesicles = 2.0 nmols/mg/sec).

Barzilai *et al.* (1984) solubilized brain synaptic plasma membrane vesicles with cholate in the presence of purified brain phospholipids and reconstituted Na+/Ca2+ exchange activity by dialysis. After sucrose gradient centrifugation of reconstituted vesicles, exchange activity was enriched ~70-fold (native synaptic membranes = 25 nmols/mg/5 min; reconstituted vesicles = 34 nmols/mg/5 min; sucrose gradient fraction = 2396 nmols/mg/5 min; note that these activities are based on 5 minute assays as opposed to the usual one second assay). Activity appeared to correlate with a 70 kDa band on SDS-PAGE. Antibodies directed against the 70 kDa band also recognized a 33 kDa band and immunoprecipitated 53-82% of the activity (Barzilai *et al.*, 1987). These results, to some extent, appear to

be in agreement with those of Philipson *et al.* (1988) and with those reported in chapter III in which Na$^+$/Ca^{2+} exchange activity correlates with a protein of 70-75 kDa. We attribute this protein, however, to breakdown of a larger parent exchanger protein of 140 kDa (chapter IV). Furthermore, Yip *et al.* (1991) used polyclonal antibodies developed against the canine cardiac sarcolemmal Na$^+$/Ca^{2+} exchanger (Philipson *et al.*, 1988) to show cross-reactivity with protein bands of 70, 120 and 160 kDa on western blots of rat brain synaptic plasma membrane proteins solubilized with decylmaltoside. In collaboration with Blaustein and associates, similar results were obtained with polyclonal antibodies developed in this laboratory (unpublished data). These data suggest that the cardiac and brain Na$^+$/Ca^{2+} exchangers are identical, but does not preclude the possibility that other proteins manifesting Na$^+$/Ca^{2+} exchange could exist in the central nervous system (see below).

Cook and Kaupp (1988) combined DEAE-Fractogel-TSK chromatography and AF Red Fractogel-TSK chromatography with either Sepharose CL-4B gel filtration or concanavalin A affinity chromatography to purify exchange activity from retinal rod outer segments. The AF Red Fractogel-TSK column was used to bind the cGMP-gated cation channel, a major contaminant at this stage, while Na$^+$/Ca^{2+} exchange activity was not bound. Final purification resulted in 108-fold enrichment with 21% recovery of Na$^+$/Ca^{2+} exchange activity which appeared to be associated with a 220 kDa band on reducing SDS-PAGE. They reported a specific

activity of 8166 nmols/mg/min. Assuming initial velocity conditions, this equates to 136 nmol/mg/sec for the ROS exchanger versus 3766 for the exchanger purified from cardiac tissue (see chapter III). These results were later confirmed by Nicoll and Applebury (1989) in which Na^+/Ca^{2+} exchange activity was purified from bovine retinal rod outer segments and was found to correlate with a single band of ~215 kDa upon SDS-PAGE. Activity reported for their most enriched preparation was also quite low relative to that obtained from cardiac tissue (Philipson et al., 1988; Ambesi et al., 1990; Ambesi et al., 1991a; see chapter III; Durkin et al. , 1991).

The lower activities observed in purified preparations of the ROS Na^+/Ca^{2+} exchanger relative to those from cardiac tissue supports the idea that the Na^+/Ca^{2+} exchanger protein may vary from tissue to tissue. Further evidence comes from the recent cloning of the cDNA for the bovine retinal rod outer segment Na^+/Ca^{2+} exchanger (Cook et al. , 1991). While "topological similarities" between the ROS Na^+/Ca^{2+} exchanger and the cardiac Na^+/Ca^{2+} exchanger were reported, the two proteins possess no amino acid homology (Cook et al., 1991).

Since activities enriched from noncardiac membrane preparations are low in comparison to those from cardiac tissue, Na^+/Ca^{2+} exchange may reflect a variation in the expression of the exchanger protein, and/or modulators of exchange activity, from tissue to tissue. Alternatively, Na^+/Ca^{2+} exchange may reflect a family of functionally similar proteins coded by completely different genes (as in the case of the cardiac and ROS Na^+/Ca^{2+} exchangers) and/or by different

genes which exhibit some degree of homology. Indeed, we have attempted to detect Na^+/Ca^{2+} exchange protein on immunoblots of canine kidney microsomal preparations (chapter IV), cultured bovine pulmonary artery endothelial cells, and cultured rat thoracic aorta vascular smooth muscle cells. Although there have been reports of Na^+/Ca^{2+} exchange activity being measured in these cell types (kidney: Lorenzen *et al.*, 1985; Smith *et al.*, 1991; cultured bovine endothelial cells: Hansen and O'Donnell, 1991; rat aorta vascular smooth muscle cells: Ashida and Blaustein, 1987), only trace amounts, at most, of the Na^+/Ca^{2+} exchange protein were detected. The data indicate that (1) too little exchanger protein was applied to the gel (suggesting low exchanger density relative to cardiac tissue) or (2) the Na^+/Ca^{2+} exchanger in these tissues is sufficiently different from the cardiac exchanger such that antibodies were unable to recognize the exchanger from these tissues.

Conversely, evidence based on immunoblots of rat synaptic plasma membranes (Yip *et al.*, 1991) and immunofluorescence microscopy (see below) clearly indicates that the protein responsible for Na^+/Ca^{2+} exchange in heart is present in neuronal tissue.

LOCALIZATION OF Na+/Ca²⁺ EXCHANGE IN NEURONAL TISSUE BY IMMUNOFLUORESCENCE MICROSCOPY

The polyclonal antibodies developed by the author against the canine cardiac Na^+/Ca^{2+} exchanger are currently being used by Blaustein and associates to localize the Na^+/Ca^{2+} exchanger in neuronal cells by immunofluorescence microscopy (Luther *et al.*, 1991). These antibodies have been shown to recognize proteins in rat synaptic plasma membranes on immunoblots comparable to those observed in canine, bovine, porcine and rat cardiac membrane preparations. Through the use of immunofluorescence microscopy, the antiserum (but not preimmune serum) was found to react intensely and selectively at the neuromuscular junctions (identified by tetramethylrhodamine-α-bungarotoxin binding) in frozen cross sections of adult rat diaphragm muscle. Furthermore, the antiserum reacted with neuronal axons in fixed and detergent-permeabilized *Xenopus* nerve and muscle co-cultures. The most intense labeling was found on the presynaptic terminals of the neuromuscular junction where the nerve had been separated from the muscle.

These studies support the hypothesis that the cardiac and neuronal Na^+/Ca^{2+} exchangers are identical (unlike the Na^+/Ca^{2+} exchanger from the outer segments of retinal rod cells) and that Na^+/Ca^{2+} exchange in nerve terminals may

be functionally important in Ca^{2+} homeostasis. Evidence supporting Ca^{2+} efflux as the primary role of Na^+/Ca^{2+} exchange in cardiac muscle leads one to suspect that Na^+/Ca^{2+} exchange in nerve terminals may play a similar role. Subsequent to Ca^{2+} influx and neurotransmitter release, Ca^{2+} efflux via the Na^+/Ca^{2+} exchanger may provide the signal necessary to terminate transmission.

FUTURE STUDIES

Now that the cDNA for the cardiac Na^+/Ca^{2+} exchanger has been cloned (Nicoll *et al.*, 1990) and purification schemes have been developed (Philipson *et al.*, 1988; Ambesi *et al.*, 1991c; Durkin *et al.* , 1991), a number of questions can be addressed. Among the first is whether the active exchanger is multimeric (see chapter III). Bifunctional cross-linking reagents could be used to determine if the active exchanger consists of a dimer or higher order of the 140 kDa protein or possibly involves as yet unidentified subunits. Undoubtedly, site-directed mutagenesis will play a fundamental role in addressing many structure/function questions. For example, through site-directed mutagenesis, sites involved in the ion translocation pathway, as well as the site involved in the regulation of Na^+/Ca^{2+} exchange by intracellular Ca^{2+}, may be identified. In addition, it may be possible to answer questions concerning the mechanism of exchange. For example, is Na^+/Ca^{2+} exchange carried out by a consecutive (Na^+ and Ca^{2+}

transported sequentially) or by a simultaneous mechanism (Na^+ and Ca^{2+} transported at the same time)?

Sites of phosphorylation and glycosylation can be determined, thereby facilitating studies addressing the regulation of exchange activity.

With DNA probes constructed from the cloned cDNA, questions such as tissue specificity and levels of expression in various tissues can be addressed, ultimately determining whether or not the Na^+/Ca^{2+} exchanger consists of a number of functionally similar proteins encoded by distinctly different genes or genes that posses some homology.

When sufficient amounts of the exchanger are purified, either from whole tissue or from cells over-expressing the exchanger, structural information may be determined with nuclear magnetic resonance spectroscopy. With the advances being made in crystallography, it may ultimately be possible to generate high quality crystals and obtain high resolution X-ray crystal structures of the Na^+/Ca^{2+} exchanger. Determination of the 3-dimensional structure could lead to the design of more selective, high affinity ligands which may be used to assess the physiological significance of Na^+/Ca^{2+} exchange in the cardiac cell and possibly in other cell types through pharmacological approaches.

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