Sodium/Calcium Exchanger: Influence of Metabolic Regulation on Ion Carrier Interactions

REINALDO DIPOLO AND LUIS BEAUGE´

Laboratorio de Permeabilidad Ionica, Centro de Biofı́sica y Bioquı́mica, Instituto Venezolano de Investigaciones Cientı́ficas, Caracas, Venezuela; and Laboratorio de Biofı́sica, Instituto de Investigaci´ın Médica "Mercedes y Martin Ferreyra", Consejo Nacional de Investigaciones Cientı́ficas y Tecı́nicas, Córdoba, Argentina

I. Discovery, Evolution of the Field in Time, and Physiological Relevance 155
II. Techniques Used to Unravel the Sodium/Calcium Exchange Transport Properties 157
III. Sodium/Calcium Countertransport: Modes of Operation, Stoichiometry, Electrogenicity, and Voltage Dependence 158
IV. Properties of Sodium and Calcium Transport Sites of the Sodium/Calcium Exchanger 161
A. External and internal sodium transport sites 161
B. External and internal calcium transport sites 161
C. Effect of nontransported monovalent cations on Na\(^{+}/Ca^{2+}\) Transport Activity 162
D. Na\(^{+}\)-Ca\(^{2+}\) interactions at the external and internal transport sites 164
E. Turnover rates, \(V_{\text{max}}\), and density of the Na\(^{+}/Ca^{2+}\) exchangers 165
V. Structural Characteristics of the Sodium/Calcium Exchange Protein 165
A. Topology, the \(\alpha-1\) and \(\alpha-2\) repeats, the intracellular loop, and the exchanger isoforms 165
VI. Regulation of the Sodium/Calcium Exchanger 166
A. Ionic regulation 166
B. Metabolic regulation 175
VII. Summary: Conclusions on the Relative Efficiency of the Calcium Extrusion Mechanisms in Squid Axons 197

DiPolo, Reinaldo, and Luis Beauge´. Sodium/Calcium Exchanger: Influence of Metabolic Regulation on Ion Carrier Interactions. *Physiol Rev* 86: 155–203, 2006; doi:10.1152/physrev.00018.2005.—The Na\(^{+}/Ca^{2+}\) exchanger’s family of membrane transporters is widely distributed in cells and tissues of the animal kingdom and constitutes one of the most important mechanisms for extruding Ca\(^{2+}\) from the cell. Two basic properties characterize them. 1) Their activity is not predicted by thermodynamic parameters of classical electrogenic countertransporters (dependence on ionic gradients and membrane potential), but is markedly regulated by transported (Na\(^{+}\) and Ca\(^{2+}\)) and nontransported ionic species (protons and other monovalent cations). These modulations take place at specific sites in the exchanger protein located at extra-, intra-, and transmembrane protein domains. 2) Exchange activity is also regulated by the metabolic state of the cell. The mammalian and invertebrate preparations share MgATP in that role; the squid has an additional compound, phosphoarginine. This review emphasizes the interrelationships between ionic and metabolic modulations of Na\(^{+}/Ca^{2+}\) exchange, focusing mainly in two preparations where most of the studies have been carried out: the mammalian heart and the squid giant axon. A surprising fact that emerges when comparing the MgATP-related pathways in these two systems is that although they are different (phosphatidylinositol bisphosphate in the cardiac and a soluble cytosolic regulatory protein in the squid), their final target effects are essentially similar: Na\(^{+}/Ca^{2+}/H^{+}\) interactions with the exchanger. A model integrating both ionic and metabolic interactions in the regulation of the exchanger is discussed in detail as well as its relevance in cellular Ca\(^{2+}\) homeostasis.

I. DISCOVERY, EVOLUTION OF THE FIELD IN TIME, AND PHYSIOLOGICAL RELEVANCE

From the classical works of Ringer in 1883 (225) and Daly and Clark in 1921 (67) it has been shown that the contraction of the frog cardiac muscle is directly related to the extracellular calcium concentration ([Ca\(^{2+}\)]\(_{o}\)), and inversely associated with the extracellular sodium concentration ([Na\(^{+}\)]\(_{o}\)). The first observations were made by Willbrandt and Koller in 1948 (256) who found that contractility of the cardiac muscle was related to the ratio [Ca\(^{2+}\)]\(_{o}\)/[Na\(^{+}\)]\(_{o}\). They proposed a site, whereby sodium
prevents calcium entry into the cardiac cell. Ten years later, Lüttgau and Neidergerke (169) confirmed these findings and concluded that calcium competes with external sodium for external sites carrying negative charges (Ca-carrier, and Na-carriers). The scheme of Ca\(^{2+}\) movements in the frog heart proposed by Lüttgau and Neidergerke (169) was not a Na\(^{+/H^{+}}\)/Ca\(^{2+}\)/H\(^{+}\) exchanger but rather a Na\(^{+/H^{+}}\)-Ca\(^{2+}\)/H\(^{+}\) antagonism. In later developments, Reuter and Seitz (223, 224) working in heart, and the Cambridge group lead by Baker (15), working in squid giant axons, documented for the first time the presence of a Na\(^{+/H^{+}}\)/Ca\(^{2+}\)/H\(^{+}\) countertransport system. The first group, by looking at \(^{45}\)Ca\(^{2+}\)/H\(^{+}\) efflux in guinea pig auricles, discovered that calcium exit was greatly reduced when external sodium and calcium were eliminated from the external medium proposing an exchange of 2 Na\(^{+}\) for 1 Ca\(^{2+}\). The second group, working in squid axons, found a ouabain-insensitive Na\(^{+/H^{+}}\) efflux incompatible with the operation of the Na\(^{+/K^{+}}\) pump (15, 16) and discovered ouabain-insensitive Na\(^{+}\)/Ca\(^{2+}\) and Na\(^{+}\)/Ca\(^{2+}\) exchanges that we know now correspond to two modes of operation of the Na\(^{+/Ca^{2+}}\) exchanger. Contrary to the 2Na:1Ca stoichiometry proposed by Reuter and Seitz (224), Blaustein and Hodgkin (46) suggested a higher stoichiometry. From that time it was clear that there existed a new transport mechanism sufficiently general to encompass different evolutionary systems such as vertebrates and invertebrates. This has been confirmed in most animal cells (for review, see Ref. 43).

The study of the Na\(^{+}/Ca^{2+}\) exchanger has experienced a rapid growth since then. Until the late 1970s, most works were related to thermodynamic and kinetic mechanisms of the Na\(^{+}/Ca^{2+}\) exchanger including apparent affinities for transported and nontransported ions (see below), as well as the discovery of two fundamental regulatory sites: the intracellular calcium regulatory site (72) and the nucleotide (ATP) site (12). The reasons for the large increase in the interest in the Na\(^{+}/Ca^{2+}\) exchange field in the last 30 years, however, come mainly from three interconnected factors: 1) the development of powerful electrophysiological techniques for the study ion transport across plasma membranes, including the patch clamp for measuring ionic currents in single cells and the giant excised patch for looking at small currents generated by carrier-mediated electrogenic ion transporters; 2) the molecular biology approach for deducing the structure-function relationships of the exchanger (see below); and 3) the progressive finding of the involvement of the Na\(^{+}/Ca^{2+}\) exchanger in several physiological functions (see Fig. 1), including cardiac muscle relaxation, control and refilling of the sarcoplasmic reticulum (SR) calcium content in the heart and in the endoplasmic reticulum (ER) of neuronal and nonexcitable cells, control of neurosecretion, excitation-contraction (E-C) coupling, and photoreception (31, 32, 36–39, 41; for review, see Refs. 6, 43, 173, 213, 28, 104). Significant attention has been focused on its role in the physiology of the heart in which 20–25% of the calcium-induced contraction results from the entrance of extracellular Ca\(^{2+}\) through the L-type Ca channels (28), whereas 75–80% is due to Ca\(^{2+}\) released from the SR. Then, for the heart [Ca\(^{2+}\)] to be in steady state, the same amount released by the SR must be pumped back into the SR, and the remaining 20% must be extruded from the cell by the exchanger and the Ca\(^{2+}\) pump. By using rapid cooling to affect

---

**Fig. 1. Intracellular Ca\(^{2+}\) homeostasis in a model cell. Role of the Na\(^{+/Ca^{2+}}\) exchanger under physiological and physiopathological conditions.**
Ca\(^{2+}\) release from the SR during contraction, Bers and Bridge (29) provided a good example of how these compete for intracellular Ca\(^{2+}\). The cross-talk between the plasmalemmal Na\(^+/H^+\)/Ca\(^{2+}/H^+\) exchanger and the intracellular Ca\(^{2+}\) stores (ER) indicates that the exchanger has a major role in control of the amplification of the Ca\(^{2+}\) induced by calcium entry through voltage-dependent calcium channels and activation of calcium-induced calcium release (CICR) from the ER. In hippocampus neurons and amacrine cells, the activity of the exchanger, in particular in its forward mode, has been shown to control the amount of calcium in the ER and, therefore, the amplification of the calcium signal. This, therefore, explains the role of the exchanger not only in E-C coupling, but also in the control of the neurosecretion processes (42, 43, 133). Furthermore, the Na\(^+/Ca^{2+}\) exchanger, working in the extrusion mode, has been recently implicated in the modulation of Ca\(^{2+}\) signal in mast cells (7). Finally, there are numerous reports that link the Na\(^+/Ca^{2+}\) exchanger with many pathophysiological syndromes including heart arrhythmias, all salt-dependent hypertension, reperfusion injury, and cardiac ischemia, among others (34, 35, 40, 43, 173, 185, 193, 238, 241, 255).

II. TECHNIQUES USED TO UNRAVEL THE SODIUM/CALCIUM EXCHANGE TRANSPORT PROPERTIES

Early studies on the Na\(^+/Ca^{2+}\) exchanger made important contributions but were limited by the use of an isolated single-cell preparation like injected squid axons (16), or whole cardiac cells (156). Mainly, they lacked control of intracellular medium, making it very difficult to study intracellular ligands, ionic and metabolic substrates that may influence the exchange activity (Ca\(^{2+}/Na^+,\) high-energy compounds, lipids, etc.). Figure 2 summarizes some of the new techniques. Figure 2A shows a diagram of the intracellular dialysis first introduced by Brinley and Mullins in 1967 (50) to study the Na\(^+/K^+\) pump in squid axons and, later, the Na\(^+/Ca^{2+}\) exchanger including voltage-clamp conditions (95). With the use of highly permeable porous cellulose acetate capillaries, it was possible to control the intracellular medium accurately allowing measurements of cation influx and efflux by means of radioactive isotopes like \(^{45}\)Ca, \(^{22}\)Na, and \(^{36}\)Rb. Its main advantage was that it preserved cytosolic structures and soluble proteins. More recently, with the development of dialysis capillaries with higher molecular weight cut off...
of all modes of transport of the Na\(^{+}/\)Ca\(^{2+}\) exchanger (see below). Another key advantage of intracellular dialysis is that it allows measurement of soluble proteins that may be involved in the Na\(^{+}/\)Ca\(^{2+}\) exchange modes that cannot be followed with electrophysiological techniques. Dialysis permits exploration of their kinetic properties and voltage dependence. A major disadvantage is that it cannot be used to measure fast kinetics such as Na\(^{+}\)-dependent inactivation or putative gating exchange currents related to the activation of the Na\(^{+}/\)Ca\(^{2+}\) exchanger by transported and regulatory ions.

Another important technique for the study of the Na\(^{+}/\)Ca\(^{2+}\) exchanger introduced by Reeves and Sutko in 1979 (219) is the plasma membrane vesicles (see Fig. 2B). This approach is widely used today to correlate biochemical and transport events in purified membrane vesicles from many biological systems. Cardiac muscle cells are the richest sources of the Na\(^{+}/\)Ca\(^{2+}\) exchanger (213) compared with other tissues such as smooth and skeletal muscle fibers, which have an activity 10 times lower (243). With the advent of whole cell voltage clamp under controlled intracellular conditions (perfusion or dialysis), many properties of the Na\(^{+}/\)Ca\(^{2+}\) exchanger became accessible, in particular its voltage dependence and ionic regulations (see below). Moreover, this technique has been proven essential in the detection of Na\(^{+}/\)Ca\(^{2+}\) exchange in many single cells, allowing investigations of its role in intracellular calcium homeostasis. Intracellular perfusion of cardiac myocytes and neurons during whole cell patch clamp has produced important progress in understanding the exchanger function (43); nevertheless, the control of the cytoplasmic environment is insufficient for many purposes. For instance, the conventional membrane patch method is not suitable for the study of non-unitary currents like those present in ion pumps and carrier transport systems due to the small ratio of surface area under the patch to that of the unspecific leak currents. This was circumvented by the development of the inside-out excised giant patch (25–30 \(\mu\)m diameter) in cardiac cells (120, 123), which allows the study of kinetics and regulatory processes related to the Na\(^{+}/\)Ca\(^{2+}\) exchanger function. One main disadvantage is that during the formation of the giant patch, cell components (among them soluble proteins that may be involved in the Na\(^{+}/\)Ca\(^{2+}\) exchange regulation) are eliminated (23, 94).

Finally, substantial progress has been made in the molecular biology of the Na\(^{+}/\)Ca\(^{2+}\) exchanger (204). Exons coding for different regions of the intracellular loop have been used in different combinations and in different tissues. New exchangers have been cloned, and several alternative splicings have been described, which has led to the classification of the Na\(^{+}/\)Ca\(^{2+}\) exchanger superfamily (204). These studies have not only produced a picture of the primary structure of the exchanger protein (204) but also, through mutagenesis and deletions of amino acids, produced a map of specific regions responsible for the transport of Na\(^{+}\) and Ca\(^{2+}\) and for regulation (see below). Antisense oligonucleotides have been useful to inhibit the Na\(^{+}/\)Ca\(^{2+}\) exchange activity in myocytes and other preparations. Finally, genetic manipulations, like transgenic mice with overexpression or knock out of the exchanger, provided elements for the understanding of its physiological and pathological significance (119, 204).

### III. SODIUM/CALCIUM COUNTERTRANSPORT: MODES OF OPERATION, STOICHIOMETRY, ELECTROGENICITY, AND VOLTAGE DEPENDENCE

Early work in squid axons indicated that the Na\(^{+}/\)Ca\(^{2+}\) exchanger can produce net calcium fluxes into or out of the cell depending on the electrochemical gradients for Na\(^{+}\) and Ca\(^{2+}\) (43). Figure 3 is a simplified consecutive kinetic model that shows the four basic modes of operation of the exchanger (80): 1) forward or direct mode, responsible for net Ca\(^{2+}\) extrusion (Na\(^{+}/\)Ca\(^{2+}\) exchange); 2) the reverse exchange, responsible for Ca\(^{2+}\) entry (Na\(^{+}/\)Ca\(^{2+}\) exchange); 3) the homologous Ca\(^{2+}/\)Ca\(^{2+}\) exchange; and 4) the homologous Na\(^{+}/\)Na\(^{+}\) exchange (for more information concerning the evidence for a consecutive or “Ping-Pong” mechanism of Na\(^{+}\) and Ca\(^{2+}\) translocation vs. a simultaneous transport model, see Ref. 43).

Numerous efforts have been made to determine the coupling ratio between Na\(^{+}\) and Ca\(^{2+}\) during the translocation process (stoichiometry). This has not been an easy task, since only the fluxes through the exchanger are relevant. Several studies demonstrated that the Na\(^{+}/\)Ca\(^{2+}\) exchange is voltage sensitive and consistent with a stoichiometry greater than 2 Na\(^{+}\) for 1 Ca\(^{2+}\). The first direct measurements of the coupled fluxes between Na\(^{+}\) and Ca\(^{2+}\) were made by T. F. Stevens and P. F. Lederer using voltage clamp (109, 120, 123) and a technique named “inverted cell voltage clamp” (120, 123), which allows the study of kinetic properties.

![Fig. 3. Consecutive kinetic scheme of transport modalities of the Na\(^{+}/\)Ca\(^{2+}\) exchanger. The carrier sites phasing the intracellular medium are labeled E\(_i\), and those phasing the extracellular solutions are labeled E\(_o\). Curved arrows indicate forward (Na\(^{+}/\)Ca\(^{2+}\)) exchange, reverse (Na\(^{+}/\)Ca\(^{2+}\)) exchange, Na\(^{+}\)/Ca\(^{2+}\) exchange. Broken arrows indicate Na\(^{+}/\)Na\(^{+}\) and Ca\(^{2+}/\)Ca\(^{2+}\) exchanges.](http://physrev.physiology.org/)
Ca\(^2+\) were carried out in ATP-depleted internally dialyzed squid axons (44). The ratio between Na\(^+\)-dependent Ca\(^2+\) efflux and Ca\(^2+\)-dependent Na\(^+\) efflux was 3.1:1. In barnacle muscle fibers and squid axons under ATP-fueled conditions, the stoichiometry was found to be also 3:1 (55, 208). Perhaps the most clear demonstration of the 3 Na\(^+\) for 1 Ca\(^2+\) stoichiometry was established by Reeves and Hale in 1984 (217) in cardiac sarcolemmal vesicles by using a null-point method to find out the [Na\(^+\)], [Ca\(^2+\)], and membrane potential at which there was no net carrier-mediated flux of calcium. With the whole cell perfused patch-clamp technique in guinea pig cardiac myocyte, Kimura et al. (155) found that the reversal potential of the Na\(^+\)/Ca\(^2+\) exchanger was very close to the value expected for a coupling ratio of 3Na\(^+\) to 1Ca\(^2+\). These determinations were later confirmed by Ehara et al. (106). Similarly, Bridge et al. (49) compared the integral of the [Ca\(^2+\)] in the inward exchange current in cardiac myocytes during depolarization, concluding that the stoichiometry was again consistent with a 3:1 coupling ratio. A direct measurement of the coupling ratio was obtained in perfused barnacle muscle fibers by measuring the Na\(^+\)-dependent \(^{45}\)Ca\(^2+\) influx and Ca\(^2+\)-dependent \(^{22}\)Na\(^+\) efflux as a function of the [Na\(^+\)], where the ratio of the two fluxes was 3:1 over the whole range of activation by intracellular Na\(^+\) (211). These measurements of coupling ratio by employing different methods lead to the conclusion that the cardiac/neuronal Na\(^+\)/Ca\(^2+\) has a coupling ratio of 3Na\(^+\) for 1Ca\(^2+\) whether the exchanger is operating in the forward or reverse mode. More recently, this subject has been revisited by Fujioka et al. (109), who take the view that precise measurements of the reversal potential of the exchanger are difficult as a consequence of accumulation and depletion of ions near the plasma membrane (106, 194). Although this problem seems to be small in the giant excised membrane patches, the above authors argue that the uses of “bleb” membrane instead of intact cardiac sarcolemmal could alter the Na\(^+\)/Ca\(^2+\) exchange properties (109). By developing large inside-out patches from intact ventricular cells, they reexamined the reversal potential of the Na\(^+\)/Ca\(^2+\) exchanger and found a variable Na\(^+\):Ca\(^2+\) stoichiometry ranging from 3:1 to near 5:1. On the other hand, Dong et al. (101) reported a 4:1 ratio for NCX.1 in transfected HEK cells. Further experiments are needed to resolve this controversial point. An interesting new finding by Kang and Hilgemann (147) using ion-selective electrodes to measure ion fluxes in cardiac giant patches is that ion flux ratio is ~3:1 during maximal transport rates in either direction and that with Na\(^+\) and Ca\(^2+\) in both sides of the patch, net current can reverse at different membrane potential. They proposed that the NCX1 can transport not only 3Na\(^+\) or 1Ca\(^2+\) but also 1Ca\(^2+\) with 1Na\(^+\) at a lower rate. These small but new transport modes might be significant in determining the background current and also the resting [Ca\(^2+\)] in the heart.

In 1979 Mullins (183, 184) demonstrated theoretically that if the energy store in the inward [Na\(^+\)] gradient (assuming a 3:1 stoichiometry) was greater than that in the [Ca\(^2+\)] inward gradient, then Ca\(^2+\) extrusion via the exchanger should be thermodynamically favored. In mathematical terms

\[ n(E_{Na} - V_m) > 2(E_{Ca} - V_m) \]

where \( n \) is the coupling ratio and \( E_{Na} \) and \( E_{Ca} \) are the equilibrium potentials for Na\(^+\) and Ca\(^2+\), respectively. These thermodynamic equations also indicate that under equilibrium conditions the ratio of free extra-to intracellular Ca\(^2+\) is

\[ \frac{[\text{Ca}^2+]_o}{[\text{Ca}^2+]_i} = \left( \frac{[\text{Na}^+]_o}{[\text{Na}^+]_i} \right)^n \times \exp(-(n - 2)V_m F/RT) \]

On the other hand, the amplitude of the exchange current (\( I_{Na/ Ca} \)) and the real contribution of the exchanger to the resting [Ca\(^2+\)] are subject to kinetic limitations. The voltage dependence of a current generated by a channel or a transporter is not a thermodynamic but a kinetic parameter that involves assumptions about the constant electrical field. Quantitative models of \( I_{Na/ Ca} \) have been proposed (30, 68, 120, 127, 129) in which the net \( I_{Na/ Ca} \) is the difference between the unidirectional calcium fluxes

\[ I_{Na/ Ca} = k_{Na/ Ca} (\text{Ca}^2+\text{influx} - \text{Ca}^2+\text{efflux}) \]

This leads to more elaborate equations that have been used to fit experimental data on current-voltage relationships of the \( I_{Na/ Ca} \)

\[ I_{Na/ Ca} = k_{Na/ Ca} \left[ ([\text{Na}^+]_o/([\text{Na}^+]_i)^r \times \exp(rV_m F/RT) \right] - \left[ ([\text{Na}^+]_o/([\text{Na}^+]_i)^r \times \exp(-(1 - r)V_m F/RT) \right] \]

where \( k_{Na/ Ca} \) is a scaling factor and \( r \) represents the position of a single energy barrier present in the electric field across the membrane and is responsible for the steepness and symmetry of the voltage dependence (68). Electroneutral fluxes associated with Na\(^+\)/Na\(^+\) and Ca\(^2+\)/Ca\(^2+\) exchanges do not produce net charge movements and cannot be measured with voltage-clamp tech-
niques. However, this does not mean that these homologous modes are voltage insensitive; in fact, the consecutive model developed by Eisner and Lederer (107) shows that Ca$^{2+}$/Ca$^{2+}$ exchange can be voltage sensitive. With the use of dialyzed squid axons to measure unidirectional isotope fluxes of Na$^+$ and Ca$^{2+}$, it has been possible to determine the voltage sensitivity of the homologous Na$^+$/Na$^+$ and Ca$^{2+}$/Ca$^{2+}$ exchanges. Figure 4, A and B, summarizes several experiments showing that the voltage sensitivity of the squid exchanger under symmetrical ionic conditions is identical for the Na$^{2+}$/Ca$^{2+}$, Na$^{+}$/Ca$^{2+}$, and Ca$^{2+}$/Ca$^{2+}$ exchanger modes, while the homologous Na$^{+}$/Na$^+$ translocation is voltage insensitive (79). A similar finding has been reported in barnacle muscle fibers (209).

More recently, by using high-resolution electrophysiological methods for measuring fast and small Na$^+$/Ca$^{2+}$ exchange currents, it has been possible to measure the charge movements associated with the activity of the Na$^+$/Ca$^{2+}$ exchanger (118, 123). In these experiments, a concentration jump of Na$^+$ or Ca$^{2+}$ induces the current generated by half of the exchange reaction cycle. Figure 4, C and D, shows the transient current generated by a Na$^+$ and Ca$^{2+}$ in oocytes expressing NCX1. The rationale for these experiments is that, in the absence of both Na$^+$ and Ca$^{2+}$ at the intracellular face, and in the presence of either Na$^+$ or Ca$^{2+}$ in the pipette, most of the carriers will be empty and facing the inner side waiting for an ion to be translocated. The application of the concentration jump (Na$^+$ or Ca$^{2+}$) at the inner side allows the translocation of either sodium or calcium to the outer face (half reaction cycle). If the translocation results in a net charge movement (electrogenic reaction cycle), a transient current will be generated. Figure 4C shows that a Na$^+$ jump induces a transient outward current. Figure 4D shows that a Ca$^{2+}$ jump does not induce any current. A likely explanation is that in NCX1 the sodium, but not the calcium, reaction is electrogenic with a binding of three Na$^+$ to a carrier carrying two negative charges. Surprisingly, Figure 4, E and F, shows that the contrary is ob-

![FIG. 4. A and B: influence of membrane potential on the different components of the Na$^+$/Ca$^{2+}$ exchange from 16 different squid axons. A: Na$^{2+}$/Ca$^{2+}$ and Ca$^{2+}$/Ca$^{2+}$ exchanges. B: Na$^{+}$/Ca$^{2+}$ and Na$^{+}$/Na$^+$ exchanges. In A, solid symbols, Ca$^{2+}$-dependent Ca$^{2+}$ efflux (Ca$^{2+}$/Ca$^{2+}$ exchange) in symmetrical Li$^+$ media; open symbols, Ca$^{2+}$-dependent Ca$^{2+}$ efflux in symmetrical Tris solutions; half open, half solid symbols, Na$^+$-dependent Ca$^{2+}$ efflux (forward exchange) in symmetrical Na$^+$ solutions. In B, solid symbols, Ca$^{2+}$-dependent Na$^+$ efflux (reverse exchange) in symmetrical Li$^+$ solutions; half open, half solid symbols, Na$^+$-dependent Na$^+$ efflux (Na$^+$/Na$^+$ exchange) in symmetrical Na$^+$ solutions. Each symbol represents a different axon. Notice that all modes of operation of the Na$^+$/Ca$^{2+}$ exchange except the Na$^+$/Na$^+$ exchange are voltage sensitive. [From DiPolo and Beauge (79) by copyright permission of the Rockefeller University Press.] C and E: charge movements associated with half reaction cycles of the Na$^+$/Ca$^{2+}$ exchange. Na$^+$ is present in the pipette and Na$^+$ is applied to the bath as indicated. D and F: Ca$^{2+}$ is present in the pipette applied to the bath; no Na$^+$ is present. Note that for the cardiac exchanger NCX1, current transients are associated with Na$^+$ translocation (C) but not with Ca$^{2+}$ translocation (D). Strikingly, the opposite is true of the squid exchanger NCX-SQ1. In this case, the electrogene step is Ca$^{2+}$ translocation (D) not Na$^+$ translocation (F). See text for details. [From He et al. (118) by copyright permission of the Rockefeller University Press.]


served in oocytes expressing the squid exchanger (NCX-SQ1), suggesting that the voltage-sensitive step is in the Ca\(^{2+}\)-dependent and not in the Na\(^{+}\) transporting branch. These species differences may explain the voltage insensitivity of the Na\(^+/\)Na\(^{+}\) exchange reaction in two invertebrates, squid (see Fig. 4B) and barnacle (209). These differences in the charge movement of the cardiac and the squid exchanger may provide an important tool for the understanding of the biophysical basis of the electrogenericity of the Na\(^+/\)Ca\(^{2+}\) exchanger.

IV. PROPERTIES OF SODIUM AND CALCIUM TRANSPORT SITES OF THE SODIUM/CALCIUM EXCHANGER

The Na\(^+/\)Ca\(^{2+}\) exchanger is highly regulated (see below); therefore, any discussion about activation kinetics by the transported ions, sodium and calcium, must necessarily take into account all ionic and metabolic modulations (see below and Table 3). In this section we review the main characteristics of sodium and calcium transport sites; we compare different preparations, but emphasize differences and similarities of two preparations in which the Na\(^+/\)Ca\(^{2+}\) exchanger has been most extensively studied: the cardiac myocyte and the squid giant axon.

A. External and Internal Sodium Transport Sites

In practically all preparations where the effects of transporting Na\(^{+}\) have been investigated (Na\(^{+}\)-dependent Ca\(^{2+}\) efflux; forward exchange, Na\(^{+}\)-dependent Na\(^{+}\) efflux; Na\(^{+}\)/Na\(^{+}\) exchange, and Na\(^{+}\)-dependent Ca\(^{2+}\) influx; reverse exchange), the activation curves show a sigmoid dependence with [Na], with a Hill coefficient close to 3 (43, 214). The \(K_{Na}\) values are 13–30 mM in cardiac sarcoplasmic vesicles (146, 200, 220, 251, 252), 34–59 mM in guinea pig atria (145), 50–80 mM in dialyzed squid axons containing ATP (70, 33), and 60 mM in barnacle muscle fibers (159, 211). A typical external Na\(^{+}\) activation curve of the forward exchange currents in single ventricular cells of guinea pig in Figure 5 has a \(K_{m}\) of 70 mM (155) and a Hill coefficient of 3. In the heart, the \(K_{0.5}\) for Na\(^{+}\) activation of Ca\(^{2+}\) transport is lower at the intracellular site (reverse exchange) than that of the external one (forward exchange) by a factor of \(\sim 8\). Conversely, in squid axons, the \(K_{0.5}\) for Na\(^{+}\) activation of Ca\(^{2+}\) influx is 50–60 mM (72), which is comparable to that of the external Na\(^{+}\) sites (33). Similarly, in barnacle muscle fibers, the \(K_{0.5}\) is \(\sim 30\) mM, only slightly less than that for outside Na\(^{+}\) (211), thus indicating no asymmetry in the sodium affinity sites in invertebrates compared with vertebrates (43). In the way this exchanger works, Na\(^{+}\) activates transport when present on membrane sides opposite to that of Ca\(^{2+}\) (forward or reverse exchange) or Na\(^{+}\) (Na\(^{+}\)/Na\(^{+}\) exchange) and inhibits when present on the same side (16, 44, 113, 224). Inhibition is competitive with a Hill coefficient close to 2 (220). In terms of its translocating sites, a remarkable property of the NCX and NCKX superfamily of exchangers is that no other monovalent cation can substitute for Na\(^{+}\). This contrasts with the Na\(^{+}\)/Ca\(^{2+}\) exchanger of mitochondria where Li\(^{+}\) can be transported as efficiently as Na\(^{+}\) (66). An exchanger different from the NCX and NCKX that catalyzes active Li\(^{+}\)/Ca\(^{2+}\) countertransport has been recently found in pancreas, skeletal muscle, and stomach (197).

At least in the squid, the binding of Na\(^{+}\) to the exchanger is not affected by the membrane potential. In squid axons internally dialyzed under voltage-clamp conditions, \(K_{0.5}\) for Na\(^{+}\) stimulation of Ca\(^{2+}\) efflux is the same at −50 mV or 0 mV transmembrane potential (79). A similar result was obtained for the Na\(^{+}\) activation of the Na\(^{+}\)/Na\(^{+}\) exchange mode (unpublished results).

B. External and Internal Calcium Transport Sites

The measurement of the apparent affinity of the extra- and intracellular Ca\(^{2+}\) transport sites are complicated for several reasons: 1) the existence of a nontransported Ca\(^{2+}\)-regulatory site on the cytosolic side (see below); 2) the difficulty in separating the apparent affinities of extra- and intracellular Ca\(^{2+}\) sites in isolated membrane vesicles that consist of a mixed population of inside-out and right-side-out vesicles, i.e., intra- and extracellular sites are simultaneously exposed to the extravesicular solution (200); and 3) the use of strong Ca\(^{2+}\) buffering agents in the perfusate could affect the apparent \(K_{Ca}\) of the Na\(^+/\)Ca\(^{2+}\) exchanger (200). Nevertheless, if one looks at preparations in which the ambiguities of sidedness are minimized, such as dialyzed squid axons, perfused barnacle muscle fibers, voltage-clamp perfused cardiac myocytes, and cardiac giant excised patches, a remarkable difference is found in the exchanger’s apparent affinities for Ca\(^{2+}\) at intra- and extracellular sites of the membrane. In squid axons fuelled with ATP, the \(K_{m}\) values are \(\sim 1–2\) \(\mu\)M for Ca\(^{2+}\) and 3,000 \(\mu\)M for Ca\(^{2+}\) (43). In embryonic chick hearts, the \(K_{m}\) is more than 100-fold higher than the cytoplasmic \(K_{m}\) (251). Current measurements in guinea pig cardiac myocytes provided values of 0.6 and 1,400 \(\mu\)M for the intra- and extracellular \(K_{0.5}\) for Ca\(^{2+}\). These data indicate that, depending on the experimental conditions, the affinities for Ca\(^{2+}\) at the inward and outward faces of the transport sites of the Na\(^+/\)Ca\(^{2+}\) exchanger differ by a factor of 10–100 (33, 79, 252, 181). In squid axons, the intra- and extracellular Ca\(^{2+}\) dependence of exchange activity was measured under otherwise symmetrical ionic and voltage conditions (79); the ratio of the \(K_{m}\) values for extra- and intracellular calcium transport...
sites is close to 10, thus indicating an asymmetry of the intra- and extracellular Ca\(^{2+}\) transport sites.

The binding of Ca\(^{2+}\) to its external transport sites is also voltage insensitive in terms of the lack of effect of membrane potential on the apparent affinity for Ca\(^{2+}\). This has been clearly demonstrated in dialyzed squid axons under symmetrical ionic conditions (79). In contrast to the high selectivity of the sodium sites for Na\(^{+}\), other divalent cations can bind to the calcium sites and be translocated in exchange for either Na\(^{+}\) or Ca\(^{2+}\) (33). In dialyzed squid axons, the order of the external apparent affinity follows the sequence Ca\(^{2+}\)/Sr\(^{2+}\)/Ba\(^{2+}\)/Mn\(^{2+}\). In sarcolemmal vesicles, the order of effectiveness in stimulating Ca\(^{2+}\) efflux in the absence of external Na\(^{+}\) in the medium was Cd\(^{2+}\)/Sr\(^{2+}\)/Ca\(^{2+}\)/Ba\(^{2+}\)/Mn\(^{2+}\) (247); as pointed out above, in this preparation there is no way to establish what sites they correspond to. The use of Ba\(^{2+}\)/Sr\(^{2+}\)/Cd\(^{2+}\) as a Ca\(^{2+}\) substitute has been nicely exploited to measure properties of the Na\(^{+}\)/Ca\(^{2+}\) exchanger in cell populations expressing the NCX (62, 246).

**C. Effect of Nontransported Monovalent Cations on Na\(^{+}\)/Ca\(^{2+}\) Transport Activity**

In both mammalian heart and squid axons, all Na\(^{+}\)/Ca\(^{2+}\) exchange modalities in which external Ca\(^{2+}\) is the
transported species (\(\text{Ca}^{2+}/\text{Ca}^{2+}\) and reverse exchange) monovalent cations, including \(\text{K}^+\) (at constant membrane potential), strongly activate the exchanger by increasing the affinity of the \(\text{Ca}^{2+}\) transport site for \(\text{Ca}^{2+}\) (4, 11, 243). This is shown in Figure 6A, where the \(\text{Ca}^{2+}\)-activated \(\text{Na}^+\) efflux (reverse exchange) is plotted against external \([\text{Ca}^{2+}]\) in the presence of \(\text{Li}^+\) or \(\text{N}-\text{methylglucamine (NMG)}^+\). Two points should be stressed: 1) the large activation of \(\text{Ca}^{2+}\) entry in the presence of \(\text{Li}^+\) but not NMG and 2) the increase in the apparent affinity for \(\text{Ca}^{2+}\) in the presence of the monovalent cation \(\text{Li}^+\). The order of effectiveness of monovalent cations follows the sequence

\[
\text{Li}^+ > \text{NMG}^+ > \text{K}^+ > \text{Na}^+.
\]

**FIG. 6.** A: effect of \([\text{Ca}^{2+}]_o\) on \(\text{Na}^+\) efflux in axons injected with (solid symbols) or without (open symbols) intracellular \(\text{Na}^+\) in the presence (circles) or absence (triangles) of external \(\text{Li}^+\). B: effect of external \(\text{Na}^+\) or \(\text{Li}^+\) on \(\text{Ca}^{2+}\) influx in injected squid axons. [From Baker et al. (16).] C: effect of external \(\text{K}^+\) on the \(\text{Na}^+_o\)-dependent \(\text{Ca}^{2+}\) influx (reverse \(\text{Na}^+/\text{Ca}^{2+}\) exchange) at constant 0 mV membrane potential. D: effect of internal \(\text{Ca}^{2+}\) on the \(86\text{Rb}\)-labeled \(\text{K}^+\) influx in the presence of 50 mM external \(\text{K}^+\). Notice that activation of the exchange by \(\text{Ca}^{2+}\) does not promote \(\text{Rb}^+\) influx. [From Condrescu et al. (64), with permission from Elsevier.] E: relative size and voltage dependence of outward \(\text{Na}^+\)/\(\text{Ca}^{2+}\) exchange current in the absence of extracellular \(\text{Na}^+\) depends importantly on the \(\text{Na}^+\) substitute; 0.5 mM \(\text{Ca}^{2+}\)-activated current in the presence of 145 mM \([\text{Na}^+]_o\) (open circles), and after replacing all of the \(\text{Na}^+\) with 145 mM \(\text{N}-\text{methylglucamine (NMG)}\) (solid circles). Free \([\text{Ca}^{2+}]_m = 50 \text{nM}; [\text{Na}^+]_m = 100 \text{mM};\) cell capacitance = 180 pF. F: 0.25 mM \(\text{Ca}^{2+}\)-activated current in the presence of 145 mM \([\text{Na}^+]_o\) (open circles), and after replacing all of the \(\text{Na}^+\) with 145 mM \(\text{Li}^+\) (solid circles). Free \([\text{Ca}^{2+}]_m = 50 \text{nM}; [\text{Na}^+]_m = 100 \text{mM};\) cell capacitance = 102 pF. [From Gadsby et al. (112), copyright 1991 New York Academy of Sciences, USA.]
Li\(^+\) > K\(^+\) = Rb\(^+\) NH\(_4\)\(^+\) > NMG\(^+\). Figure 6B shows that even Na\(_o\)\(^+\), at low concentrations, activate Ca\(^{2+}\) influx; obviously, the dose-response curve for Na\(_o\)\(^+\) is biphasic due to displacement of Ca\(_o\)\(^{2+}\) from the external transporting sites at high [Na\(^+\)]\(_o\) (9). Interestingly, the Na\(_o\)\(^+\)-dependent Ca\(^{2+}\) flux (forward) is not affected by either K\(_o\)\(^-\) (at constant membrane potential) (5) or high concentrations of Li\(_o\)\(^+\) (which do not affect the membrane potential) (10), i.e., there is no chemical action of external monovalent cations on the exchange activity when Na\(_o\)\(^+\) is the transported species (10). At present, it is not known whether the generation of the monovalent site occurs after Ca\(_o\)\(^{2+}\) binds to the carrier, or if the site exists independently of the binding of Ca\(_o\)\(^{2+}\). The experiments on exchange activity in vesicles can be interpreted by a model (243) including two external binding sites. A divalent site (A) which can bind a single calcium ion or two sodium ions and a second monovalent cation site (B). When the predominant extracellular ion is Na\(^+\), all A and B sites will be occupied by this ion, and therefore, no monovalent cation effect will take place. The physiological importance of the external monovalent cation sites is also unknown since, under normal physiological conditions, the monovalent cation site, if it indeed exists (site B), is occupied by external Na\(^+\). Finally, by a still unknown reason, little effect of nontransported monovalent cations was found in cardiac giant patches (174).

In cardiac vesicles and squid axons, the activating monovalent cations are not cotransported during Ca\(^{2+}\) translocation. This is illustrated in the experiments of Figure 6, C and D. In Figure 6C, with the membrane potential clamped at 0 mV, K\(_o\)\(^-\) reversibly increases the Na\(_o\)\(^+\)-dependent Ca\(^{2+}\) influx. That this increase is reverse mode exchange is proven by its disappearance upon removal of Ca\(^{2+}\). In Figure 6D, also at 0 mV membrane potential, the [Ca\(^{2+}\)]\(_i\), that activates reversed exchange in the presence of K\(^+\) does not modify the (\(^{86}\)Rb)K\(^+\) influx (64). An interesting observation concerning the effect of external nontransported monovalent cations is that they seem to influence the voltage dependence of the Na\(^+\)/Ca\(^{2+}\) exchanger. Although in squid axons the matter is still controversial (4), in dialyzed cardiac ventricular myocytes from guinea pig there is a noticeable difference between the outward Na\(^+\)/Ca\(^{2+}\) exchange current-voltage relationship obtained in Li\(_o\)\(^+\) and that in NMG\(_o\)\(^+\). Figure 6, E and F, shows the relative size and voltage dependence of outward Na\(^+\)/Ca\(^{2+}\) exchange current when all external Na\(^+\) is replaced either by NMG\(^+\) or Li\(^+\). Compared with Li\(_o\)\(^+\) media, the size of the current decreases in NMG\(^+\) and becomes voltage insensitive, leading to the conclusion that the Na\(_o\)\(^+\)/Ca\(_o\)\(^{2+}\) exchange current is critically dependent on small monovalent cations (112). The significance of this finding is unknown, but is another aspect where further studies are needed to understand the mechanism of the overall exchange process. Finally, and in contrast to what happens in mammalian heart and squid nerve, in the NCKX of rods external K\(^+\) activates and is concomitantly cotransported with Ca\(^{2+}\) into the cell (230–233).

Intracellular nontransported monovalent cations (not including intracellular protons; see sect. viA) also stimulate the Na\(^+\)/Ca\(^{2+}\) exchanger. Intracellular K\(^+\) and Li\(^+\), at constant membrane potential, are powerful activators of the forward exchange mode (79, 96). The K\(_o\)\(^-\)/Ca\(^{2+}\)_o for K\(_o\)\(^+\) is 90 mM in dialyzed squid axons; as this activation is independent of [Na\(^+\)]\(_o\), it rules out any K\(_o\)\(^-\)-Na\(_o\)\(^+\) competition. Monovalent cation activation was explored further in diazolized squid axons by measuring the Ca\(^{2+}\)-dependent Ca\(^{2+}\) efflux in the presence and absence of intracellular Li\(^+\) with and without Li\(^+\) in the extracellular solution. An intriguing and still unexplained result was the lack of effect of intracellular Li\(^+\) when the external monovalent cation site was vacant (18); on the other hand, the exchange fluxes do not display a biphasic relationship with [Na\(^+\)]\(_o\) (as happens with the Na\(_o\)\(^+\)-dependent Ca\(^{2+}\) efflux). Actually, Na\(_o\)\(^+\) inhibits through a sigmoid dose-response curve. Perhaps the monovalent activating cation binding sites are different in the external and internal surfaces. Nevertheless, the large activation of the exchanger, in all its modes, by intracellular K\(^+\) should be considered when analyzing the physiological role of the Na\(^+\)/Ca\(^{2+}\) exchanger in cases where intracellular K\(^+\) is substituted with NMG\(^+\), Tris\(^+\), or other cations.

D. Na\(^+\)-Ca\(^{2+}\) Interactions at the External and Internal Transport Sites

Dialyzed squid axons were used to analyze Na\(^+\) and Ca\(^{2+}\) interactions at the external surface of the membrane by measuring the influx of Ca\(^{2+}\), at 0.5 and 10 mM external Ca\(_o\)\(^{2+}\), at various [Na\(^+\)]\(_o\). In agreement with results in injected squid axons (see Fig. 6B), Ca\(^{2+}\) influx as a function of [Na\(^+\)]\(_o\) is always biphasic. A model that accounts for these Na\(_o\)\(^+\)/Ca\(_o\)\(^{2+}\) interactions reproduces the data obtained in intact injected and dialyzed squid axons (18). The model assumes that all external Na\(^+\) and Ca\(^{2+}\) interactions with the Na\(^+\)/Ca\(^{2+}\) exchanger are based on Na\(^+\)/Ca\(^{2+}\) competition for the transporting site plus a positive cross-reactivity between the monovalent activating and the transporting sites. In addition, the occupancy of the external monovalent activating site by Na\(_o\)\(^+\) increases the maximal rate of exchange. The fitting of the data requires that the binding of Ca\(^{2+}\) to its transporting site increases the affinity of the monovalent cation site by about fivefold. The occupancy by Na\(_o\)\(^+\) of the monovalent activating site increases the affinity of the transport site for Ca\(_o\)\(^{2+}\) by the same magnitude. In addition, the binding of Na\(_o\)\(^+\) to the external transporting site must increase the affinity of the monovalent activating site by twofold, i.e., in the squid exchanger Na\(_o\)\(^+\) and Ca\(_o\)\(^{2+}\) interactions display competition.
together with a positive activating cross-reactivity between the monovalent activating and the transporting sites.

The interactions of Na\(^+\) and Ca\(^{2+}\) with intracellular transport sites can be adequately explained by the simple model that is basically a kinetic scheme of random equilibrium conditions with Na\(^+\) and Ca\(^{2+}\) competition (18).

E. Turnover Rates, \(V_{\text{max}}\), and Density of the Na\(^+\)/Ca\(^{2+}\) Exchangers

The Na\(^+\)/Ca\(^{2+}\) exchanger can have a very high rate of transport when it is fully activated. For instance, in rod outer segments, flux and current measurements indicate Ca\(^{2+}\) fluxes of 40–50 pmol \(\cdot\) cm\(^{-2}\) \(\cdot\) s\(^{-1}\) (231); these figures correspond to changes in \([\text{Ca}^{2+}]_i\) of >30 \(\mu\text{M/s}\). In rod axons, the \(V_{\text{max}}\) can reach values up to 20–40 pmol \(\cdot\) cm\(^{-2}\) \(\cdot\) s\(^{-1}\) (91; and unpublished results). In cardiac cells, current (155) and flux (58) measurements produced values as high as 30 pmol \(\cdot\) cm\(^{-2}\) \(\cdot\) s\(^{-1}\). Based on kinetic analysis in reconstituted proteoliposomes, an estimate turnover value of \(\sim 1,000\) s\(^{-1}\) has been obtained for the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger (50). From giant patch measurements at saturating intracellular Ca\(^{2+}\), currents of 30 pA/F were recorded for \(I_{\text{NaCa}}\) with a density of about 400 exchanger molecules/\(\mu\text{m}^2\). If the surface of the myocytes is close to \(25 \times 10^5\ \mu\text{m}^2\), then a maximum turnover rate of 5,000 s\(^{-1}\) can be estimated (130). Similarly, from channel-like noise analysis and charge movements in cardiac giant excised patches, it has been possible to detect turnover rates of \(\sim 5,000\) s\(^{-1}\) for the transport of both Na\(^+\) and Ca\(^{2+}\) using nonsaturating ion concentrations (122).

V. STRUCTURAL CHARACTERISTICS OF THE SODIUM/CALCIUM EXCHANGE PROTEIN

A. Topology, the \(\alpha-1\) and \(\alpha-2\) Repeats, the Intracellular Loop, and the Exchanger Isoforms

Since 1990, molecular biology techniques applied to the study of the Na\(^+\)/Ca\(^{2+}\) exchanger have given abundant and crucial information on its structure-function relationships. The canine cardiac NCX1 exchanger was the first to be purified (202) and cloned (1, 190). Further subtypes that are products of different genes (NCX2 and NCX3) were later found in the brain (204, 245). Also, during the biosynthesis of the carrier, an NH\(_2\)-terminal hydrophobic segment of 32 amino acids (“signal peptide”) (see Fig. 7 for details) is cleaved off to produce a mature protein (103). The full-length, mature cardiac exchanger contains 938 amino acids (204). Initially, hydropathy analysis suggested that the exchanger had 11 transmembrane segments; the NH\(_2\) terminus is glycosylated, located extracellularly, and does not seem to be involved in exchange activity (111, 132, 229). Presently, it is believed (Fig. 7) that transmembrane segment 6 is part of the large intracellular loop (light shaded cylinder) and that the old transmembrane segment 9, although hydrophobic, does not span the membrane but forms a P-loop type structure similar to the pore-forming region of ion channels. The middle of this segment 9 has a GIG sequence resembling the motif characteristic of K\(^+\) channels. Thus, in the latest version, the Na\(^+\)/Ca\(^{2+}\) exchanger contains nine transmembrane segments, five from the NH\(_2\) terminus up to the large intracellular loop and four from that loop up to the COOH terminus (65, 141, 164, 191). The large intracellular cytosolic loop has \(\sim 550\) amino acids (204). Although this loop contains important regulatory sites, it does not appear to be required for transport, since a mutant lacking most of this loop (\(\Delta 240–679\)) still retains exchange activity (177). Toward the NH\(_2\)- and COOH-terminal portions of the exchanger there are two repeat sequences of \(\sim 40\) amino acids called \(\alpha-1\) and \(\alpha-2\) repeats (Fig. 7) (236). They are conserved in all members of the NXC family and other cation exchangers (204, 236) and are thought to play an important role in ion transport. In the NCX, the \(\alpha-1\) repeat includes part of transmembrane segments 2 and 3 and a loop connecting them; the \(\alpha-2\) repeat consists of a portion of the transmembrane segment 7 and the GIN transmembrane part in the COOH-terminal region. By cysteine substitutions in these loops and the use of externally or internally applied sulfhydryl reagents, evidence has been gathered indicating that they are part of the ion translocation pathway (140, 141). Also, mutagenesis of trans-
membrane segment 2 markedly changes ion selectivity (100); as an example, substitution of threonine at position 103 with valine changes the high selectivity for Na\(^+\), resulting in an exchanger that can countertransport Li\(^+\) with Ca\(^{2+}\) (100, 189). Recently, Philipson and co-workers (195) have addressed the importance of the α-1 repeat in ion translocation through the exchanger. In a series of experiments in which mutations of the reentrant loop and transmembrane segment 3 (TMS3) were performed, Ottolia et al. (195) conclude that TMS3, and not the reentrant loop is involved in Na\(^+\) binding and transport. The Na\(^+\) binding site associated with TMS3 appears to be unique and not involved in Ca\(^{2+}\) binding. Furthermore, they found a highly specific requirement for an asparagine or aspartate residue at position 143, indicating a crucial role of Asn\(^{143}\) in Na\(^+\) translocation.

The large intracellular loop plays a critical role in the ionic and metabolic regulation of the exchanger (88, 89, 204). Mild proteolysis of the cytosolic side of the exchanger eliminates regulation by intracellular Na\(^+\), Ca\(^{2+}\), H\(^+\), and MgATP (91, 97, 123). The NH\(_2\)-terminal portion of the loop has a 20-amino acid domain rich in hydrophobic and basic amino acids and is called the XIP (exchange inhibitor peptide) region; in addition, it has an overall amino acid sequence similar to the calmodulin-binding domain (190). This region is associated with the Na\(^+\) inactivation process (see Fig. 7). Around the central zone of the loop, there is a sequence of 135 amino acids (371–508; see Fig. 7) containing highly acidic residues (two zones of three aspartyl each) that bind Ca\(_{\text{in}}\) with high affinity (163); the mutation of these aspartyl residues markedly lowers exchange activity and reduces the binding affinity for Ca\(^{2+}\), i.e., this region is responsible for the Ca\(^{2+}\)-dependent or allosteric regulation of the exchanger (178). Towards the COOH-terminal end of the intracellular loop (see Fig. 7) there is a region, encoded by six exons (A to F), responsible for the splice variants of NCX1 (157, 161, 207). At least 12 splice variants have been reported. Most excitable tissues have exchangers with exon A. Exon B predominates in other tissues (204). It is believed that a tissue-specific pattern of alternative splicing may change the kinetics of the exchanger function, such as Ca\(_{\text{in}}^{2+}\)-dependent regulation, Na\(^+\)-dependent inactivation, and ATP modulation. In this respect, in the hippocampus there is a differential, cell specific distribution of the Na\(^+\)/Ca\(^{2+}\) exchange subtypes: NCX1 is expressed in neurons but not in glial cells, whereas NCX2 is predominantly expressed in glia (245). NCX1 and NCX2 appear to be linked to a high capacity for Ca\(^{2+}\) extrusion in neurons and glia, respectively (245). Similarly, it has been found that two splice variants of the Drosophila exchanger exhibit different regulation by intracellular Ca\(^{2+}\) and Na\(^+\) (207). Preferential distribution of NCX1, NCX2, and NCX3 in different cell types is likely related to specific requirements for intracellular Ca\(^{2+}\) homeostasis, but more work is required to elucidate this point (187).

The most extensive studies of the Na\(^+\)/Ca\(^{2+}\) exchanger have been carried out in cardiac membrane (patch-clamp and membrane vesicles) and squid nerve (injected, dialyzed axons, and membrane vesicles). Therefore, it seemed pertinent to compare them. Some basic properties, like stoichiometry of the exchanging cations and regulation by Ca\(^{2+}\), Na\(^+\), H\(^+\), and ATP, are present in both system’s preparations (see Ref. 89); nevertheless, the presence of fundamental differences between them may provide insight into their structure-function relationships (see sect. viB) and will certainly facilitate the development of models of the regulatory processes that control the activity of the Na\(^+\)/Ca\(^{2+}\) countertransport. Figure 8 and Table I show the amino acid homology between squid nerve and the canine heart Na\(^+\)/Ca\(^{2+}\) exchangers (data calculated from Ref. 118). As a whole, NCX-SQ1 is 58% identical to the cardiac exchanger and possesses overall identity (41–64%) with other exchangers (118). The regions that have functional importance such as the Ca\(_{\text{i}}\)-regulatory binding domain (46% homology), Na\(^+\) inactivation (56% homology), and COOH terminal (70% homology) are well conserved. Furthermore, the α-1 and α-2 repeats involved in ion translocation are well conserved in the two preparations (see Fig. 8). There are, however, substantial functional differences, mostly related to regulation, in particular, their voltage dependence (strong voltage dependence of Ca\(^{2+}\)/Ca\(^{2+}\) but not Na\(^+\)/Na\(^+\) exchange in the squid as compared with strong voltage dependence of the Na\(^+\) translocation branch in the cardiac) and metabolic regulation (see sect. viB). Combination of molecular biological and electrophysiological methods may provide important information to understand the structure-function relationships of this superfamily of countertransport systems.

VI. REGULATION OF THE SODIUM/CALCIUM EXCHANGER

A. Ionic Regulation

1. Ca\(_{\text{i}}^{2+}\)-dependent regulation

In intact, injected squid axons, addition of a Ca\(^{2+}\) chelator such as EGTA or quin 2 into the cytosol reduced the Ca\(^{2+}\) influx and the Ca\(_{\text{i}}^{2+}\)-dependent Na\(^+\) efflux (3, 9), suggesting that Ca\(_{\text{i}}^{2+}\) could somehow regulate the exchanger. The use of dialyzed squid axons showed, unambiguously, that the Na\(^+\)-dependent Ca\(^{2+}\) influx (reverse exchange) required cytoplasmic Ca\(^{2+}\) as an essential factor (72, 92). One of these experiments is illustrated in Figure 9A. In this case, Ca\(^{2+}\) influx in the absence of Na\(^+\) is quite small ([Ca\(^{2+}\)]\(_i\) = 0.7 μM), indicating the presence...
of little Ca\(^{2+}\)/Ca\(^{2+}\) exchange (cartoon on the right in Fig. 9A). The addition of Na\(^{+}\) in the presence of Ca\(^{2+}\) in the dialysis medium produces a large reverse exchange (cartoon 2, right of Fig. 9A). The key observation is that removal of Ca\(^{2+}\) completely inhibits the reverse exchange (cartoon 3, right of Fig. 9A). Figure 9B shows data from a series of experiments in intact cardiac myocytes in which the inward Na\(^{+}\)/Ca\(^{2+}\)/Ca\(^{2+}\)/Na\(^{+}\) (reverse) exchange current is plotted against the intracellular calcium concentration (in the patch pipette); the apparent Ca\(^{2+}\)/Na\(^{+}\) affinity is 50 nM (181).

TABLE 1. Amino acids homology between squid nerve and canine heart Na\(^{+}\)/Ca\(^{2+}\) exchangers

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>58%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+}) regulatory binding domain</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>Na(^{+}) inactivation</td>
<td>60%</td>
<td></td>
</tr>
<tr>
<td>COOH terminal (97 residues)</td>
<td>70%</td>
<td></td>
</tr>
</tbody>
</table>

Calculated from He et al. (118).
for the function of the heart, since the NXC1 might play an integrating role of \([\text{Ca}^{2+}]_i\) transients over multiple beats as opposed to a beat to beat regulation (216). On the other hand, in the Drosophila melanogaster Na\(^+/\text{Ca}^{2+}\) exchanger clone (Calx), binding of \([\text{Ca}^{2+}]_i\) to its regulatory site reduces exchange activity (131). At present, it is unknown whether alternatively spliced variants of vertebrate exchangers also show this anomalous \([\text{Ca}^{2+}]_i\) modulation.

In addition to \([\text{Ca}^{2+}]_i\), other divalent cations can interact with the \([\text{Ca}^{2+}]_i\) regulatory site bringing up allosteric activation of the exchanger; this happens with Ba\(^{2+}\) and La\(^{3+}\), which are also transported. However, Ba\(^{2+}\) binds to the \([\text{Ca}^{2+}]_i\) regulatory site with much lower affinity (246), while La\(^{3+}\) does so with much higher affinity (62) than \([\text{Ca}^{2+}]_i\). More recently, it has been reported that Cd\(^{2+}\) regulates the exchange activity with a \(K_D\) in the picomolar range (158). An important question is whether \([\text{Ca}^{2+}]_i\) reg-
ulatory and transport sites are different structures. The available experimental evidence indicates that they are.

1) If one looks at the Na\(^+\)/H\(^+\) requiring transport modalities of the exchanger (Na\(^+\)/Na\(^+\) and Na\(^+\)/Ca\(^{2+}\) exchanges), Ca\(^{2+}\) is essential for their function; therefore, the Ca\(^{2+}\) regulatory site and the Na\(^+\) transport sites exist simultaneously.

2) The \(\alpha\)-1 and \(\alpha\)-2 repeats, conserved regions of most exchangers thought to participate in the formation of the ionophoric paths, are far away from the intracellular loop (204). Amino acid substitutions in these regions markedly affect transport capacity but not Ca\(^{2+}\) modulation (189).

3) PCMBS, applied internally in dialyzed squid axons, drastically reduces the affinity of the Ca\(^{2+}\) regulatory site without affecting the maximal Ca\(^{2+}\) transporting capacity (83).

The possibility that the Ca\(^{2+}\) bound to the regulatory site is also extruded during the exchange cycle has also been ruled out by experiments in dialyzed squid axons. If those Ca\(^{2+}\) were translocated, one would expect 45Ca efflux to increase together with Na\(^+\) efflux during Na\(^+\)/H\(^+\) activation of the reverse exchange (Na\(^+\)-dependent Ca\(^{2+}\) influx), but this does not happen. Figure 10 shows measurements of three of the exchange reactions of the Na\(^+\)/Ca\(^{2+}\) exchanger in dialyzed squid axons: Na\(^+\)-dependent 45Ca\(^{2+}\) influx, Na\(^+\)-dependent 45Ca\(^{2+}\) efflux, and Ca\(^{2+}\)-dependent 45Ca\(^{2+}\) efflux, all as a function of the [Na\(^+\)] at constant [Ca\(^{2+}\)] (81). As expected for a countertransport system, Na\(^+\)/Ca\(^{2+}\) and Ca\(^{2+}\)/Ca\(^{2+}\) exchanges (open symbols) are inhibited by intracellular Na\(^+\); actually, at 100 mM internal Na\(^+\), the Ca\(^{2+}\) efflux-dependent modes almost vanish. On the contrary, activation of the reverse exchange (Ca\(^{2+}\)/Na\(^+\)) by [Na\(^+\)] increases continuously until it saturates at \(\sim 300\) mM Na\(^+\), and this increase is not accompanied by any Ca\(^{2+}\) efflux. Another important inference from these experiments is that internal Na\(^+\) does not displace Ca\(^{2+}\) from its regulatory site;

### Table 2.

**Apparent affinity for Ca\(^{2+}\) (K\(_{0.5}\)) of the Ca\(^{2+}\) regulatory site of the Na\(^+\)/Ca\(^{2+}\) exchanger in different preparations**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>(K_{0.5}), nM</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barnacle</td>
<td>400–600</td>
<td>211</td>
</tr>
<tr>
<td>Squid</td>
<td>400–800</td>
<td>77, 89</td>
</tr>
<tr>
<td>Intact myocyte</td>
<td>20–200</td>
<td>109, 181</td>
</tr>
<tr>
<td>NCX1 clone</td>
<td>100–400</td>
<td>173, 215</td>
</tr>
<tr>
<td>Intact excised myocyte patch</td>
<td>100–400</td>
<td>109</td>
</tr>
<tr>
<td>Cardiac sarcolemmal vesicles</td>
<td>120–140</td>
<td>27</td>
</tr>
</tbody>
</table>

![Figure 10](http://physrev.physiology.org/). **Inhibition of Na\(^+\)/Ca\(^{2+}\)** (forward) and Ca\(^{2+}\)/Ca\(^{2+}\) exchanges and activation of Ca\(^{2+}\)/Na\(^+\) (reverse) exchange by Na\(^+\) at saturating [Ca\(^{2+}\)]. Observe that at 100 mM or higher [Na\(^+\)], forward and Ca\(^{2+}\)-Ca\(^{2+}\) exchanges are very small in contrast to a still-increasing reverse exchange (see text for details).

[From DiPolo and Beaugé (81), copyright 1991 New York Academy of Sciences, USA.]
otherwise, one should expect a bell-shaped curve between reverse Na\(^+/\)Ca\(^{2+}\) exchange and [Na\(^+\)]. Similar to the external monovalent cation modulation, the apparent affinity of the Ca\(^{2+}\) regulatory site is voltage insensitive (81).

The classical experiments of Hilgemann in giant excised sarcolemmal membrane patches (121) demonstrated that controlled chymotrypsin digestion of the cytosolic membrane side produced a fully activated exchanger, independent of [Ca\(^{2+}\)]. In Figure 11A, in a mammalian cardiac excised path, after chymotrypsin treatment, the reverse exchange current is high and is not affected by Ca\(^{2+}\). Figure 11B shows similar results with the cloned squid Na\(^+/\)Ca\(^{2+}\) exchanger (SCX-SQ1) expressed in Xenopus oocytes (118). The large intracellular hydrophilic loop of the exchanger was proposed to contain the Ca\(^{2+}\) regulatory site (177). This localization was demonstrated by Levitsky et al. (163) by measuring the binding of \(^{45}\)Ca\(^{2+}\) to that loop expressed as a fusion protein. The calcium binding domain is located near the center of the loop (amino acids 371–508). It interacts with Ca\(^{2+}\) in a cooperative manner (Fig. 12). Also notice that single site mutations of aspartates 447, 449, and 498 drastically reduce the Ca\(^{2+}\) affinity. This calcium binding domain does not have a helix-loop-helix (EF-hand) characteristic of other Ca\(^{2+}\) binding proteins (163). In addition, the binding of Ca\(^{2+}\) induces a marked shift in the SDS-PAGE mobility, indicating sizable conformational changes in the protein. Conformational changes in the Ca\(^{2+}\) regulatory domain of the mammalian heart Na\(^+/\)Ca\(^{2+}\) exchanger following the binding of Ca\(^{2+}\) have been studied recently with the FRET technique in cells where this domain was expressed with a fluorophor donor at one end and an acceptor attached to the other end (196).

Titrations of FRET efficiency as a function of [Ca\(^{2+}\)] gave a \(K_D\) for Ca\(^{2+}\) of \(~140\) nM in the absence of Mg\(^{2+}\) and 400 nM in its presence. These values are close to those reported for the apparent Ca\(^{2+}\) affinity of the Ca\(^{2+}\) regulatory site in mammalian and invertebrate cells obtained by other methods (see Table 2). These FRET experiments also demonstrate that the Ca\(^{2+}\) regulatory site of the Na\(^+/\)Ca\(^{2+}\) exchanger is able to sense changes in the cytosolic calcium during spontaneous cardiac contraction in neonatal cardiac myocytes responding to changes in Ca\(^{2+}\) in the physiological range; in addition, the conformational change of the protein is sufficiently fast to follow changes in the [Ca\(^{2+}\)], during E-C coupling (196).

2. Na\(^{+}\)-dependent inactivation

By exposing the intracellular surface to the bath medium and allowing fast changes in ion concentrations on that side, giant cardiac sarcolemmal patches provided a powerful technique to analyze the Na\(^{+}\) inactivation of the Na\(^+/\)Ca\(^{2+}\) exchanger (123). In Figure 13A, with 1 \(\mu\)M cytosolic and 4 mM extracellular Ca\(^{2+}\) and without Na\(^+\) on either side, there is no exchange current. Addition of 90 mM Na\(^+\) to the cytosolic side produces a fast development of an outward current; almost immediately, that current starts to decay exponentially (“inactivates”), with a time constant of \(~4\) s, to a steady state that represents \(~20\)% of the peak. Figure 13A also shows that Na\(^{+}\) inactivation is destroyed when the cytoplasmic side of the membrane is treated with chymotrypsin, indicating that this process takes place at the large intracellular loop. According to the model proposed by Hilgemann et al. (129) and Matsuoka et al. (179) (Fig. 13B), inactivation starts with the binding of 3 Na\(^+\) to their E\(_p\)Na intracellular transport sites; this conformation can follow two routes: either face the extracellular side, E\(_p\)Na, or become occluded into the (E\(_p\)Na) state. The conformational change E\(_p\)Na ↔ (E\(_p\)Na) is governed by two rate constants: \(k_{\text{inac}}\) and \(k_{\text{back}}\) for inactivation and recovery from inactivation, respectively (175). In native cardiac patches
and intact cardiac myocytes, intracellular Ca\textsuperscript{2+} has two effects on Na\textsuperscript{+}-dependent inactivation: in the low range of concentrations (0.01–0.5 \textmu M), the peak current is increased; at higher [Ca\textsuperscript{2+}]i (1–30 \textmu M), the Na\textsuperscript{+}-dependent inactivation is markedly reduced (Fig. 13C). The increase in the peak current has been attributed to the exchanger recovering from a Na\textsuperscript{+}-independent inactive state (I\textsubscript{2}; see model of Fig. 13B) that takes place from the Na\textsuperscript{+} free carrier (E\textsubscript{1}) (129); the attenuation of the Na\textsuperscript{+}-dependent inactivation at higher Ca\textsuperscript{2+} responds to a facilitation from the recovery of the Na\textsuperscript{+}-dependent inactivation (I\textsubscript{1}) (179).

Similar transient and steady-state exchange currents can be predicted by the schemes of Hi\textsuperscript{+}, Na\textsuperscript{+}, and Ca\textsuperscript{2+} interactions suggested by DiPolo and Beaugé (90) (see below).

A region called "XIP" (exchanger inhibitory peptide) in the NH\textsubscript{2} terminus of the large intracellular loop is related to Na\textsuperscript{+}-dependent inactivation. A synthetic peptide with an amino acid sequence similar to the XIP region completely inactivates the exchanger, with a \textit{K}\textsubscript{i} close to 100 nM, when applied intracellularly (164). Mutations of the XIP region alter Na\textsuperscript{+} inactivation (176). Mutations that eliminate inactivation produce stronger interactions between phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P\textsubscript{2}] and XIP, whereas those interactions are weakened by mutations that promote inactivation (116, 176). Mutations in the XIP peptide indicate that although its full length is required for maximal potency, the more relevant inhibitory components are basic and aromatic residues between positions 5 and 16 (117). A recent work claims that in NCX the binding site for XIP may be located in the loop zone between amino acids 562 and 679 (171); at any rate, although the site of interaction of the XIP region with the rest of the NCX molecule has not been identified, it has been suggested that this region functions as an auto inhibitory domain that shifts the active-inactive states of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger.

**FIG. 12.** The Ca\textsuperscript{2+} binding domain of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. Top: amino acid sequence of the exchanger loop region which strongly binds Ca\textsuperscript{2+}. Two acidic segments of the fragment are underlined, and amino acid residues which were examined by mutagenesis are marked by an asterisk. When the region was truncated from the NH\textsubscript{2} terminus to amino acid 385 or from COOH terminus to amino acid 494 (arrows), strong Ca\textsuperscript{2+} binding was lost. A segment truncated at the COOH terminus to amino acid 508 (thick arrow) retained strong Ca\textsuperscript{2+} binding. Bottom: Ca\textsuperscript{2+} affinities of wild-type and mutated exchanger loops. Ca\textsuperscript{2+} binding to wild-type fusion protein 240–532 and to mutations of 240–532 are as noted. 45Ca\textsuperscript{2+} binding was measured in the presence of 1 mM MgCl\textsubscript{2}. [From Levitsky et al. (163).]
3. $H^+_\text{r}$ and $H^+_\alpha$ effects on Na$^+$/Ca$^{2+}$ exchange activity

Inhibition of the Na$^+$/Ca$^{2+}$ exchanger by intracellular protons was first described in injected squid axons by Baker and McNaughton (14) and dialyzed squid axons (74) and further characterized in mouse heart (251, 252), dog cardiac sarcolemmal vesicles (201), reconstituted Na$^+$/Ca$^{2+}$ exchanger in liposomes (153), and myocyte excised patches (97, 98). In dialyzed squid axons there is an asymmetric effect of protons (Fig. 14A), whereas intracellular acidification strongly inhibits and internal alkalinization markedly enhances activity, similar changes in extracellular pH have no effect. The lack of effect of external pH in the squid exchanger contrasts with a recent report in patch-clamped guinea pig ventricular myocytes where extreme external alkalinization ($pH_\text{ext} = 10$) or acidification ($pH_\text{ext} = 5$) strongly inhibits the Na$^+$/Ca$^{2+}$ exchanger (105); to account for these results, the authors suggest that protons may interact at multiple extracellular sites of the exchanger to affect its stoichiometry.

Intracellular proton inhibition of the Na$^+$/Ca$^{2+}$ exchanger in the complete absence of Na$^+_\text{r}$ (proton inhibition per se) was first shown in giant excised membrane patches from cardiac myocytes of adult guinea pig (97). Figure 14B describes a typical experiment in which the excised patch was preincubated at two different pH values: 6.4 and 7.2, before inducing the Na$^+_\text{r}$-dependent reverse exchange current. Preexposure to pH 6.4 in the absence of Na$^+_\text{r}$ decreased the amplitude of the current, indicating that proton inhibition developed in the absence of Na$^+$. Recently, in dialyzed squid axons it has also been demonstrated that protons per se are strong inhibitors of the exchanger: reduction of 0.4 pH units from the physiological pH$_\text{r}$ of 7.3 causes 90% inhibition of the Na$^+$/Ca$^{2+}$ exchange (90). The pH$_\text{r}$ sensitivity of the cardiac Na$^+$/Ca$^{2+}$ exchanger outward current in excised cardiac patches from guinea pig (Fig. 14C) (97) is in good agreement with other studies in mouse heart cells (252), cardiac sarcolemmal vesicles (201, 243), and dialyzed squid axons (74). A remarkable similarity found in all these preparations is the steep dependency of exchange activity on intracellular protons near the physiological pH$_\text{r}$. As mentioned above, a treatment of the Na$^+$/Ca$^{2+}$ exchanger with chymotrypsin for a limited time disrupts the intracellular regulatory loop; this causes the exchanger to lose

![Figure 13](http://physrev.physiology.org/)

**Figure 13.** Secondary modulation of outward exchange current: sodium-dependent inactivation and calcium-dependent activation. A: outward Na$^+$/Ca$^{2+}$ exchange current in a giant cardiac membrane patch. *Left:* current response under standard experimental conditions. Note decay of current during application of cytoplasmic sodium. *Right:* current response after treatment of the cytoplasmic membrane face with chymotrypsin. Note attenuation of the current decay. [Redrawn from Hilgemann et al. (128).] B: minimum model of cardiac Na$^+$/Ca$^{2+}$ exchanger function. The exchanger functions in a consecutive cycle. Ion translocation takes place in two steps. The exchanger can enter an inactive state whenever binding sites facing the cytoplasmic side are loaded with three sodium ions (I$_1$) or from the empty unloaded carrier (I$_2$) (see text for detail). [From Hilgemann (125) and Matsuoka et al. (170).] C: secondary Na$^+_\text{r}$ and Ca$^{2+}$ regulation of the cardiac Na$^+$/Ca$^{2+}$ exchanger, NCX1. The *left panel* demonstrates Na$^+_\text{r}$-dependent inactivation. The *right panel* demonstrates Ca$^{2+}$ regulation. In this case the level of regulatory calcium in the bath was 15 μM instead of 1 μM. Two phenomena are evident. First, the Na$^+_\text{r}$-dependent inactivation is eliminated in the presence of high regulatory calcium. Ca$^{2+}$ modulates Na$^+_\text{r}$ regulation. Second, upon removal of regulatory Ca$^{2+}$, exchange activity declines to zero. Ca$^{2+}$ is transported by the exchanger but also exerts a separate modulatory effect. [From Philipson and Nicoll (204), copyright 2000 by Annual Reviews.]
secondary regulation by Ca\textsuperscript{2+} and Na\textsuperscript{+} (see also sect. viB). Figure 14C shows that this enzymatic digestion also dramatically reduces the effects of intracellular protons, indicating that these interactions take place on, or are related to, that loop. The exquisite sensitivity of the exchanger to pH\textsubscript{i} values around 7.2–7.4 is expected to play a significant role in its modulation under physiopathological conditions. This may explain, in part, the deregulation of the exchanger induced by chymotrypsin, which has generally been ascribed to the removal of Ca\textsuperscript{2+} - and Na\textsuperscript{+} -dependent modulation (98).

4. Ca\textsuperscript{2+}-H\textsuperscript{+} interactions

If the effects of protons involve some kind of competition with Ca\textsuperscript{2+}, that interaction might occur at two places: Ca\textsuperscript{2+} transport sites, which will render the exchanger unable to transport in the Ca\textsuperscript{2+} efflux mode, or at the Ca\textsuperscript{2+} regulatory site, inhibiting all transport modes of the exchanger. In either case, inhibition by H\textsuperscript{+} should be a function of [Ca\textsuperscript{2+}]. Experiments carried out to explore these possibilities in giant excised membrane patches from guinea pig were not conclusive (97). This point was also explored in dialyzed squid axons by using two approaches: 1) by following the Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} efflux (forward mode) without Na\textsuperscript{+} at variable pH\textsubscript{i} values as a function of the [Ca\textsuperscript{2+}] (range from 0.7 to 1,000 \textmu M) and 2) by determining the apparent affinity of the Ca\textsuperscript{2+}-regulatory site on the basis of the Ca\textsuperscript{2+} stimulation of the Na\textsuperscript{+}/Na\textsuperscript{+} exchange (see above and Fig. 15B) at different pH\textsubscript{i} values. Figure 15A demonstrates proton inhibition associated with a proton-calcium antagonism in the absence of Na\textsuperscript{+}; notice that at 1,000 \textmu M Ca\textsuperscript{2+}, protons are ineffective. An answer regarding the site(s) at which protons act is provided by the second approach. Figure 15B shows that, in the absence of ATP, the apparent affinity of the Ca\textsuperscript{2+} regulatory site decreases 60 times when pH\textsubscript{i} goes from 8.8 to 6.9. The fact that at 1,000 \textmu M Ca\textsuperscript{2+} the V\textsubscript{max} of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger is not modified by pH\textsubscript{i} strongly
suggests that $H_i^+\cdotCa^{2+}$ interactions take place at, or at least mostly at, the $Ca^{2+}$ regulatory site. Two additional lines of evidence point in that direction: 1) intracellular, controlled chymotrypsin digestion relieves proton inhibition and eliminates $Ca^{2+}$ regulation without affecting the maximum $Na^+/Ca^{2+}$ exchange rate (see Figs. 14C and 11B) and 2) PCMBs, which is known to block the $Ca^{2+}$ modulation of the squid exchanger, renders intracellular protons ineffective (89). Similar effects of protons on the apparent affinity of the $Ca^{2+}$ regulatory site have been reported in the cardiac exchanger in which intracellular alkalinization dramatically increases the affinity of that site for $Ca^{2+}$ (127, 129).

5. $Na^+/H_i^+$ synergistic inhibition: kinetic model

As shown initially by Doering and Lederer (97) in guinea pig cardiac cells under patch clamp, $H_i^+$ inhibition is enhanced by $Na^+$; this additional effect disappears 9 s after removal of $Na^+$. The model proposed for $H_i^+$ and $H_i^+-Na_i^+$ interactions with the intracellular side of the exchanger is shown in Figure 16A. Protons can bind to the free, calcium-loaded, and sodium-loaded carrier, but the affinity is higher in the sodium-bound form (99). In all cases, dead-end inhibitory complexes are formed. This model can also explain the observation that in dialyzed squid axons $Ca^{2+}/Ca^{2+}$ exchange (a $Ca^{2+}$ extruding mode) is much less sensitive to $pH_i$ than $Na^+/Ca^{2+}$ and $Na^+/Na^+$ exchanges, the two modes that move $Na^+$ outward (76). An important consequence of this $Na_i^--H_i^+$ interaction is that $Na_i^+$ inactivation observed at or near $pH$ 7.2 results from a combined synergistic interaction between $H_i^+$ and $Na_i^+$. In fact, there are similarities between $Na_i^+$-dependent inactivation and $H_i^+$-inhibition: both develop with a time course (seconds) and both are prevented by chymotrypsin digestion. A $Na_i^+-H_i^+$ synergism predicts that internal alkalinization should reduce $Na_i^+$-dependent inactivation. This is confirmed in Figure 16B, where the fast $Na_i^+$ inactivation at $pH_i$ 6.8 is completely overcome by raising internal $pH$ to 7.8 (129). Figure 16B also shows that intracellular alkalinization increases the steady-state level of the exchange current. Intracellular $H_i^+-Na_i^+$ synergism in the inhibition of the $Na^+/Ca^{2+}$ exchanger at steady state has also been observed in dialyzed squid axons. In Figure 16C, the $K_{0.5}$ for intracellular $Na^+$ inhibition goes from 10 mM at $pH_i$ 6.9 to 90 mM at $pH_i$ 8.8 (90).

6. $Ca^{2+}/H_i^+-Na_i^+$ interactions: kinetic models

The topics reviewed in section vi, A1–A5, are intended to provide a detailed analysis of how the $Na^+/Ca^{2+}$ exchanger is regulated by ions acting on regulatory sites located at the intracellular loop of the exchanger. We have intentionally emphasized the similarities and differences between the two preparations used in most of these studies: the mammalian heart and the squid nerve. The relevant findings are summarized in Table 3. There are two points to be emphasized in the models of ionic regulation presented so far. 1) $Na_i^+$ inhibits by interacting with the intracellular transport sites. This interaction of $Na_i^+$ with its transport sites is implicit in Figure 16 (99) and explicitly stated in Figure 13B (123, 129, 179, 129). 2) In the $Na_i^+$-dependent inactivation (Fig. 13B) and $H_i^+-Na_i^+$ synergism (Fig. 16A), the $Ca^{2+}$ regulatory site was left aside. To understand how the $Na^+/Ca^{2+}$ exchanger is modulated by metabolism (see sect. viB), it is necessary to take into account the three ionic interactions, $H_i^+$, $Na_i^+$, and $Ca^{2+}$, all together. Figure 17A shows a kinetic scheme that includes $H_i^+-Ca^{2+}$ competition and $H_i^+-Na_i^+$ syner-
gism as the basis for this ionic regulation (90). For simplicity, only the intracellular ionic interactions (E forms) have been considered. An important feature of this scheme, that differs from the two mentioned above, is that the inhibitory Na binding site is different from the Cai regulatory site, since Ca is not displaced from that site by Na (see above). E is the free carrier, Ca,E the carrier loaded with Ca at the regulatory site, Ca,E,Na and Ca,E,3Na are the carriers loaded with 1 Ca or 3 Na on the transporting sites ready to perform either Ca or Na extrusion. H.E, H.E,Na, and H,Na are carriers binding H and Na at their inhibitory sites. Proton inhibition occurs even in the absence of Na (HE complex). Since, in the absence of protons (extreme alkalinization), there is no Na-dependent inhibition, protons must bind first. This explains the reduction in the apparent affinity of the intracellular Ca regulatory site. Na enhances H inhibition by binding to HE to produce the HE,Na form that allows the binding of a second proton leading to the H,Na dead end complex. Therefore, inhibition of intracellular Na will have two components: H,Na synergism on the regulatory site and Na,Ca competition at the transporting sites; the second effect can be dissected from the first during internal alkalinization (see Fig. 16C). From the data, and the simulations, H,Na synergism is the major component at low [Na], whereas competition at the transport sites comes into play at high [Na]. This new model puts the Ca regulatory site at the center of the scene; as such it can explain most of the experimental data on ionic regulation of both mammalian and invertebrate exchangers. Figure 17, B and C, is a simulation of the Na/Ca exchange activity during steady state, as a function of [Ca] and [Na], at different pH values in the absence of intracellular ATP. Figure 17, D and E, shows that this new model also predicts the response of the exchanger under pre-steady-state conditions: Na inactivation and the relief of Na inactivation at high [Ca], and alkaline pH, respectively.

B. Metabolic Regulation

1. Modulation by nucleotides

A) Discovery of ATP stimulation. The first indication that there was an energy-dependent regulation of the Na/Ca exchange came from work in injected giant

**FIG. 16.** A: model of the Na/Ca exchange cycle and inhibition by cytoplasmic protons. A simple consecutive transport model is assumed with five different forms corresponding to different occupancy states of the transport site accessible only to the extracellular environment, and five forms corresponding to states with intracellularly accessible transport sites. [From Doering et al. (90), copyright 1996 New York Academy of Sciences, USA.] B: outward exchange current transient at pH 6.8 and 7.8, activated by 100 mM cytoplasmic calcium in the presence of 4 mM cytoplasmic calcium. [From Hilgemann et al. (127) by copyright permission of The Rockefeller University Press.] C: effect of Na on the forward Na/Ca exchange at different pH values in the absence of ATP. The data show the Na-dependent inhibition of forward Na/Ca exchange at different pH values in the absence of ATP. Ordinate, percent inhibition of Na/Ca exchange; abscissa, intracellular Na in millimolar. The error bars indicate SE. The mean temperature was 17°C. Notice the exquisite sensitivity of the exchange activity to Na at the acidic pH. [From DiPolo and Beaugé (90).]
axons (12) with manipulations that reduced the levels of ATP. That reduction was achieved in two ways: inhibition of ATP production with cyanide or increase in ATP hydrolysis with apyrase injections. In both cases the Na$_4$-$/$Ca$_2$+ efflux was inhibited (12). That ATP was actually involved in stimulation of the exchanger was later shown in dialyzed axons from the squid *Doryteuthis plei* where removal of ATP from the cytosol inhibited the Na$_4$-$/$Ca$_2$+ efflux. This effect was reversed by restoring the nucleotide in the dialysis solution. At the same time, these experiments showed that the requirement for ATP was not absolute, since a sizable part of the exchange fluxes was present without the nucleotide (69, 70). Although the mechanism(s) and/or the targets of that stimulation were not known then, ATP appeared to be an important, nonessential activator. ATP stimulation of Ca$_2$+ efflux has two components: the Ca$_2$+ pump and the Na$_4$-$/$Ca$_2$+ exchange. In this particular case (40 mM Na$_4$, 0.7 µM Ca$_2$+, and 3 mM MgATP), the two components are not very different. At any rate, the stimulation of the

### TABLE 3. Effects of ligand-carrier interactions with extracellular and intracellular sites of mammalian and invertebrate Na$_4$-$/$Ca$_2$+ exchangers

<table>
<thead>
<tr>
<th>Sites</th>
<th>Extracellular</th>
<th>Invertebrate nerve</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transport, ionic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca$_2$+</td>
<td>Competes with Na$_4$+ affinity: increase by monovalent cations and ATP$_p$</td>
<td>Competes with Na$_4$+ affinity: increase by monovalent cations and ATP$_p$</td>
<td>4, 5, 16, 18, 33, 45, 81, 211, 220</td>
</tr>
<tr>
<td>Sr$_2$+, Ba$_2$+</td>
<td>Substitute Ca$_2$+ with lower affinity</td>
<td>Substitute Ca$_2$+ with lower affinity</td>
<td>33, 62, 204, 247</td>
</tr>
<tr>
<td>Na$_+$</td>
<td>Competes with Ca$_4$+ affinity: increase by ATP$_p$</td>
<td>Competes with Ca$_4$+ affinity: increase by ATP$_p$</td>
<td>73, 76, 174, 181, 208, 251</td>
</tr>
<tr>
<td><strong>Nontransport, ionic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li$<em>+$, K$</em>+$</td>
<td>Activate</td>
<td>Activate</td>
<td>4, 5, 9–11, 16, 18, 75, 80</td>
</tr>
<tr>
<td>H$_+$</td>
<td>Inhibition at alkaline and acidic pH, 5 and 10</td>
<td>No effect pH, 6 to 9</td>
<td>14, 74, 97, 98, 105, 127, 201</td>
</tr>
<tr>
<td>NMG$_-$</td>
<td>Inert</td>
<td>Inert</td>
<td>16, 33, 79, 112</td>
</tr>
<tr>
<td>Choline$_+$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris$_-$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOPS$_-$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES$_-$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ni$_2$+, Cd$_2$+</td>
<td>Inhibit</td>
<td>Inhibit</td>
<td>137, 155, 180, 203, 214</td>
</tr>
<tr>
<td>La$_3$+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCB</td>
<td>Inhibit</td>
<td>Inhibit</td>
<td>237</td>
</tr>
<tr>
<td>PCMBs</td>
<td>Not studied</td>
<td>No effect</td>
<td>83</td>
</tr>
<tr>
<td>KBR-7943</td>
<td>Reversibly inhibits reverse Na$^+$/Ca$_2$+ exchange</td>
<td>Irreversibly inhibits all Na$^+$/Ca$_2$+ exchange modes</td>
<td>136, 143, 253</td>
</tr>
<tr>
<td>SEA-0400</td>
<td>Reversibly inhibits reverse Na$^+$/Ca$_2$+ exchange</td>
<td>Inhibits mainly forward Na$^+$/Ca$_2$+ exchange, ATP$_p$ protects inhibition</td>
<td>19, 48, 135, 160, 244</td>
</tr>
<tr>
<td>SN-6</td>
<td>Reversibly inhibits mainly reverse Na$^+$/Ca$_2$+ exchange</td>
<td>Not studied</td>
<td>135</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sites</th>
<th>Intracellular</th>
<th>Invertebrate nerve</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transport, ionic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca$_2$+ (regulatory)</td>
<td>Essential for all transport modes</td>
<td>Essential for all transport modes</td>
<td>72, 76, 127, 155, 181</td>
</tr>
<tr>
<td>Ba$_2$+, Cd$_2$+</td>
<td>Replace Ca$_2$+ at the regulatory site</td>
<td>Replace Ca$_2$+ at the regulatory site</td>
<td>158, 215</td>
</tr>
<tr>
<td>La$_2$+</td>
<td>Inhibitory; reduce affinity of Ca$_2$+ regulatory site</td>
<td>Inhibitory; reduce affinity of Ca$_2$+ regulatory site</td>
<td>14, 74, 80, 97, 98, 127, 153</td>
</tr>
<tr>
<td>H$_+$</td>
<td>Favor proton inhibition</td>
<td>Favor proton inhibition</td>
<td>89, 98</td>
</tr>
<tr>
<td>Na$_+$</td>
<td>Activates</td>
<td>Activates</td>
<td>4, 5, 18, 33, 81, 96</td>
</tr>
<tr>
<td>Li$<em>+$, K$</em>+$</td>
<td>Required for ATP stimulation</td>
<td>Required for ATP stimulation,</td>
<td>&lt;1 mM: required for ATP stimulation, &gt;1 mM: inhibits ATP stimulation</td>
</tr>
</tbody>
</table>

*Physiol Rev* • VOL 86 • JANUARY 2006 • www.prv.org
Na⁺-dependent exchange component is about sevenfold. The $K_{1/2}$ values for ATP are different, $\sim 20 \, \mu M$ for the pump and $250 \, \mu M$ for the exchanger (33, 70, 73).

In early experiments with sarcolemmal membrane vesicles, Caroni and Carafoli (56) reported an ATP modulation that took place via a calmodulin-dependent kinase-mediated phosphorylation step. In that report the authors proposed the existence of an associated Ca²⁺-stimulated phosphatase. On the other hand, work done by others failed to detect any ATP effects on the Na⁺/Ca²⁺ exchanger in mammalian heart vesicles (205, 213). With the introduction of the giant patch technique (61, 126), an MgATP stimulation of the reversal exchange mode was demonstrated in guinea pig, rabbit, and mouse myocytes. The $K_m$ for MgATP was exceedingly high, $\sim 12 \, \mathrm{mM}$. In the late 1990s, a consistent MgATP stimulation of the uptake of Ca²⁺ through the Na⁺/Ca²⁺ exchanger was demonstrated in bovine heart sarcolemmal vesicles, but to detect it, addition of vanadate to the extravesicular solution was required (27).

### Table 3—Continued

<table>
<thead>
<tr>
<th>Sites</th>
<th>Mammalian heart</th>
<th>Invertebrate nerve</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Stimulates, $K_{0.5} 0.2-5 , \text{mM}$, protects against Na⁺ inhibition, favors binding of external Na⁺ and Ca²⁺ to their transport sites, phosphoinositide metabolism is involved (PIP₂), regulation does not require a cytosolic regulatory protein</td>
<td>Stimulates, $K_{0.5} 0.25 , \text{mM}$, protects against Na⁺ inhibition, favors binding of external Na⁺ and Ca²⁺ to their transport sites, requires a soluble cytosolic regulatory protein (SCRP)</td>
<td>8, 12, 23, 25–27, 70, 73, 77, 94, 120, 166, 167, 188, 222</td>
</tr>
<tr>
<td>Hydrolyzable ATP analogs</td>
<td>Mimics ATP</td>
<td>Mimics ATP</td>
<td>26, 27, 71, 82, 118</td>
</tr>
<tr>
<td>Nonhydrolyzable ATP analogs</td>
<td>No effect</td>
<td>No effect</td>
<td>26, 27, 71, 82, 118</td>
</tr>
<tr>
<td>ITP, UTP, CTP</td>
<td>Not studied</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Chromium-ATP</td>
<td>Not studied</td>
<td>Inhibits MgATP effect</td>
<td>75, 82</td>
</tr>
<tr>
<td>ADP</td>
<td>Not studied</td>
<td>Inhibits only in the presence of ATP</td>
<td>75</td>
</tr>
<tr>
<td>P₆, PnPP</td>
<td>Not studied</td>
<td>Stimulates only in the presence of ATP without affecting PA regulation</td>
<td>85, 87, 91</td>
</tr>
<tr>
<td>Phosphoarginine</td>
<td>No effect</td>
<td>Stimulates preferential forward exchange, no effect on the Ca⁴, regulatory site, increase affinity of Ca⁴ transport site, no phosphorylation of the carrier involved</td>
<td>85, 87, 91</td>
</tr>
<tr>
<td>Protein kinases (PKA, PKC)</td>
<td>Stimulate in whole cells</td>
<td>No effect</td>
<td>85, 87, 91</td>
</tr>
<tr>
<td>PKC activators (phorbol esters)</td>
<td>Stimulate in whole cells</td>
<td>No effect</td>
<td>61, 87, 138, 139, 142, 226, 227</td>
</tr>
<tr>
<td>Protein phosphatases</td>
<td>No effect</td>
<td>Inhibits both ATP and PA regulation</td>
<td>27, 61, 87</td>
</tr>
<tr>
<td>PIP₃, PIP₂</td>
<td>Stimulate</td>
<td>No effect</td>
<td>8, 21, 27, 93, 118, 125</td>
</tr>
<tr>
<td>Antibody against PIP₂</td>
<td>Inhibits</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>PI-PLC</td>
<td>Inhibits</td>
<td>No effect</td>
<td>61, 87, 138, 139, 142, 226, 227</td>
</tr>
<tr>
<td>Protein kinase inhibitors</td>
<td>Not studied</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>PCMBS</td>
<td>Not studied</td>
<td>Decrease affinity of Ca²⁺ regulatory site, blocks ATP regulation</td>
<td>83</td>
</tr>
<tr>
<td>Polylsines</td>
<td>Inhibits</td>
<td>No effect</td>
<td>61, 87</td>
</tr>
<tr>
<td>XIP</td>
<td>Inhibits with and without ATP</td>
<td>Inhibits with and without ATP</td>
<td>27, 60, 84, 116, 117</td>
</tr>
<tr>
<td>FMR-Fa peptide</td>
<td>Inhibits</td>
<td>Inhibits</td>
<td>84, 150–152</td>
</tr>
<tr>
<td>SEA-0400</td>
<td>Not studied</td>
<td>Inhibits mainly forward mode, favors Na⁺ inactivation and proton inhibition. Antagonized by ATP</td>
<td>19</td>
</tr>
</tbody>
</table>
cells, both with native exchanger and when expressing a cloned exchanger (Table 3).

B) KINETIC EFFECTS. MgATP modulation takes place by affecting the binding affinity of several ligands. The initial findings came from studies in squid axons on the forward exchange mode. On the one hand, there was an increase in the apparent affinity for the external Na\textsuperscript{+}/H\textsuperscript{+} transport site, from 120–160 mM to 50 mM (12, 13, 33, 70) and for intracellular Ca\textsuperscript{2+}, 1–3 M to 1–3 M (33, 75). On the other, an apparent increase in the \( V_{\text{max}} \) of the forward mode was also reported, but that happened only in the presence of intracellular Na\textsuperscript{+} (71, 75, 222) and was reduced as the [Ca\textsuperscript{2+}] increased (33). MgATP stimulation was also seen on the reverse \( \text{Na}^+/\text{Ca}^{2+} \) modes (75, 77). The initial proposal that ATP antagonizes Na\textsuperscript{+} inhibition of the forward exchange by decreasing the affinity for Na\textsuperscript{+} (33, 222) was unsatisfactory, since a corresponding

**FIG. 17.** A: kinetic model of Na\textsuperscript{+}-H\textsuperscript{+}-Ca\textsuperscript{2+} interactions. B: kinetic model simulations for the Na\textsuperscript{+}, H\textsuperscript{+}, and Ca\textsuperscript{2+} interactions in the regulation of the squid Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in the absence of ATP. B and C are steady-state simulations (90), and D and E are pre-steady-state simulation (unpublished data). See text for details.
change in the $K_{1/2}$ for ATP at high [Na$^+$], was not observed (75). The ATP-dependent increase in the apparent affinity for the binding of Ca$^{2+}$ to its intracellular regulatory site (77) focused for the first time on the possible interrelationship between intracellular ionic and ATP modulation of the exchanger.

In cardiac giant excised patches MgATP diminishes the extent and slows the kinetics of Na$^+$ inactivation (Fig. 18A) (61, 128). Figure 18B shows that removal of MgATP causes an increase in the rate of Na$^+$ inactivation and a decrease in the steady-state exchange current. Consequently, the nucleotide decreases the affinity for Na$^+$. In addition, the nucleotide acts with low affinity, since 10 mM almost doubles the effect of 2 mM (Fig. 18C). There is a second effect: reduction of the rundown time constant of the exchanger activity after intracellular Ca$^{2+}$ is removed (see Fig. 18, E and F). A reduction in the “off” rate constant for Ca$^{2+}$ binding is equivalent to an increase in the affinity of that site for Ca$^{2+}$. This relationship between MgATP and the Ca$^{2+}$ regulatory site was confirmed and extended in bovine cardiac sarcosomal vesicles where MgATP increases the affinity of that site for Ca$^{2+}$ by ~5- to 10-fold (27). Actually, antagonism between Na$^+$ and Ca$^{2+}$ had already been observed (129), but at that time there was no information about the possible nature and significance of that connection. Another important observation regarding the mammalian cardiac exchanger was that the characteristics of the MgATP up-regulation are maintained when the exchanger is expressed in an heterologous cell like Xenopus oocytes (179). This has at least two relevant connotations. On the one hand, the final target for that modulation is indeed the exchanger protein and, on the other hand, the biochemical machinery involved is present both in mammalian and amphibian cells.

The work reviewed above documents the modulation of the Na$^+/\text{Ca}^{2+}$ exchanger by the coupled interactions of nontransport sites with Na$^+$, H$^+$, and ATP (43, 88, 124), but some of the effects were analyzed individually. Recent experiments in squid axons made substantial advances on the role played by MgATP on the overall picture of intracellular cation modulation (89, 90). On the one hand, MgATP prevents the synergistic intracellular proton-sodium inhibition of the exchanger (Fig. 19, A and B). On the other hand, the presence of MgATP is comparable to an intracellular alkalization, since at pH 8.8 the exchanger is already stimulated and the nucleotide is no longer effective (Fig. 19C). Although the intimate mechanism is unknown, this result is consistent with the lack of ATP stimulation in the absence of intracellular Na$^+$ (222). Actually, the inhibition seen at high Na$^+$ concentration at pH 8.8 and no ATP can be fully accounted for by the competition between Na$^+$ and Ca$^{2+}$ for the intracellular transport sites. Figure 20, A and B, illustrates the effects of [Ca$^{2+}$]i on Na$^+-\text{Na}^+$ exchange through the Na$^+/\text{Ca}^{2+}$ exchanger. Because intracellular Ca$^{2+}$ regulatory and transport sites coexist, this approach, together with experiments on reversal exchange, is particularly suitable for exploring possible modifications on the Ca$^{2+}$ regulatory site alone. These experiments show the dramatic counter effect of MgATP proton-induced reduction on the affinity of the Ca$^{2+}$ regulatory site. At pH 6.9 the $K_{0.5}$ for Ca$^{2+}$ is 200 $\mu$M, 50-fold higher than the 4 $\mu$M observed at pH 7.3; addition of 3 mM MgATP at pH 6.9 reduces the $K_{0.5}$ for Ca$^{2+}$ to 4 $\mu$M (Fig. 20B). These and other results led to the proposal that upregulation of the squid Na$^+/\text{Ca}^{2+}$ exchanger by MgATP occurs through a reduction in the apparent affinity for H$^+$ and Na$^+$ binding to nontransport inhibitory sites. An “ATP regulatory region” has therefore been added (surrounded by dotted lines in Fig. 21) to the model of Na$^+-\text{H}^+$ and Ca$^{2+}$ interactions described above (see Fig. 17). The actual effects of the nucleotide are to minimize or prevent the formation of the H.E$_1$, H.E$_1$.Na, and H.E$_2$.Na complexes by reducing the affinities for H$^+$ and Na$^+$ binding. Obviously, although the model does not elaborate on the underlying metabolic pathway, it presupposes that the final effects take place on the intracellular regulatory loop of the exchanger protein. Simulations carried out with this model predict practically all Ca$^{2+}$-Na$^+$-H$^+$ and ATP interactions observed for the Na$^+/\text{Ca}^{2+}$ exchanger in dialyzed squid axons. Five of them are illustrated here. Figure 21A and B, shows that whereas at acidic pH MgATP produces a marked upregulation of the exchanger, at an alkaline pH of 8.8 the nucleotide is practically ineffective. Figure 21C displays a differential sensitivity to H$^+$ in the absence and presence of MgATP and also illustrates the lack of nucleotide stimulation of the exchanger at alkaline pH. Figure 21D shows the model prediction of a marked increase in the affinity the Ca$^{2+}$ regulatory site for Ca$^{2+}$ when MgATP is included in a dialysis solution at a pH of 6.9. As a comparison, it also shows the high affinity that the Ca$^{2+}$ regulatory site when bathed in an alkaline solution (pH 8.8). As indicated in Figure 21E, the proposed kinetic scheme accounts for the prevention of Na$^+$ inactivation by MgATP under pre-steady-state conditions. In that simulation, a Na$^+$ concentration of 40 mM and an internal pH of 6.9 were used. Notice that in the presence of 3 mM ATP the initial peak of transient activation is increased, the transient inhibition is reduced, and the steady-state current is augmented.

C) BASIC CHARACTERISTICS OF ATP-REGULATED TRANSPORT SYSTEMS: MECHANISMS UNDERLYING ATP STIMULATION OF SODIUM/CALCIUM EXCHANGE. Two enzyme systems requiring ATP and Mg$^{2+}$ can be the basis for, or be associated with, cation transport across biological membranes: 1) transport ATPases, which are indeed the translocating structure and are fueled by the free energy derived from ATP hydrolysis, or 2) kinases, which may regulate translocation via phosphorylation of the transporter molecule or through the phosphorylation of associated regula-
tory structures. Many transport ATPases are also called P-ATPases because during the cycle they form a phosphorylated intermediate in an aspartyl residue. The cycle starts with the phosphorylation of the enzyme in the presence of Mg\(^2\+\) and the intracellular translocating cation. Free ADP is then released, but Mg\(^2\+\) stays attached to the enzyme as longs as it remains phosphorylated (see Ref. 53). In the case of kinases, after phosphorylation of the substrate, ADP is released as a MgADP complex (102). The kinase-phosphorylated substrate may lose its phosphate spontaneously, but usually dephosphorylation is accelerated by Mg\(^2\+\)-activated phosphatases.

The basic feature, common to all Na\(^+/\)H\(^+\)/Ca\(^2+/\)H\(^+\) exchange preparations, is that the simultaneous presence of Mg\(^2\+\) is essential for the onset of the ATP upregulation (61, 75). The \(K_m\) for ATP is variable: \(\sim 250 \text{ M}\) in squid nerve (73), 500 \(\text{ M}\) in mammalian sarcolemmal heart (27) and nerve (26) vesicles, and in the millimolar range in giant patches of mammalian heart (27). The fractional increase in exchange fluxes varies with the preparations and also, as will be seen below, with the experimental conditions. Depending on the preparation, there are also additional requirements for ligand interactions. In dialyzed squid axons, where the four exchange modes are all stimulated by MgATP, stimulation is increased by intracellular vanadate (\(K_{1/2} \sim 10 \text{ M}\)) and millimolar concentrations of inorganic phosphate and \(p\)-nitrophenylphosphate (75). The mammalian heart exchanger in native cells, or even expressed in heterologous cells, under patch-clamp conditions is stimulated by MgATP; mammalian heart sarcolemmal or brain vesicles require the addition of 100–500 \(\text{ M}\) vanadate (26, 27). Upon removal of ATP, in the presence of Mg\(^2\+\), upregulation of the exchangers is lost (93). In excised giant sarcolemmal patches, the effect of MgATP is insensitive to the protein kinases inhibitors 1-(5-iso-

**FIG. 18.** Analysis of MgATP effect on sodium-dependent inactivation kinetics. Results are with 2 mM calcium and 150 mM sodium in the pipette. Current transients were recorded for cytoplasmic solution switches from a solution containing sodium-free cytoplasmic solution with 3 \(\mu\text{M}\) free calcium to one with 100 mM sodium and 3 \(\mu\text{M}\) free calcium, and back to the original solution. Dotted lines give the current level in the absence of cytoplasmic calcium and sodium. A: records were first obtained in the presence of 2 mM MgATP. B: ATP was then washed out and the current transients became more pronounced over 20 min. C: finally, 10 mM ATP was applied and the current transients became much less pronounced. D: the slow recovery portions of the records upon switching back to sodium-free solution were fitted to exponentials and are plotted semi-logarithmically after subtracting an asymptote. The rate constants for rundown with 0 mM, 2 mM, and 10 mM ATP are 0.05, 0.18, and 0.34 s\(^{-1}\), respectively. E: application and removal of 2 \(\mu\text{M}\) free calcium in the presence of 100 mM cytoplasmic sodium. Note that the initial rate of rise of current upon application of calcium is almost unchanged by MgATP, while the decay rate upon removal of calcium is drastically slowed. F: decay of outward exchange current upon removal of cytoplasmic calcium before and after MgATP. An asymptote was subtracted and results are plotted semi-logarithmically. [From Hilgemann et al. (127) by copyright permission of The Rockefeller University Press.]
quinaline-sulfonyl)-2-methylene-piperazine or to inhibitor peptides of calmodulin-dependent protein kinase II, PKC, and cAMP-dependent protein kinases. Because it was also insensitive to alkaline, acid, and heart extracted protein phosphatases, it was concluded that stimulation of the exchanger did not involve protein kinases (61). This concurs with results in mammalian heart sarcolemmal vesicles where MgATP stimulation is insensitive to alkaline protein phosphatase (27).

D) NUCLEOTIDE SELECTIVITY. The different properties of nucleotides polyphosphate can be related to 1) the nucleoside structure, 2) the capability to be hydrolyzed, 3) the number of phosphates, and 4) the addition of other elements (for instance, sulfur or chromium) to the phosphate chain. Furthermore, selectivity studies can provide important clues as to what metabolic path or paths are likely to be involved. This approach was used in the ATP stimulation of the Na\(^+\)/Ca\(^{2+}\) exchanger.

I) Nucleoside structure. In squid axons stimulation of the exchanger is highly selective towards adenosine,

![Figure 19](http://physrev.physiology.org/)

**FIG. 19.** ATP relief of Na\(^+\)/H\(^+\) inhibition of forward Na\(^+\)/Ca\(^{2+}\) exchange. A: Na\(^+\)-induced inhibition of Na\(^+\)-dependent Ca\(^{2+}\) efflux at pH 6.9 in an axon dialyzed first without ATP, and after 3 mM ATP. Notice the following: 1) the large activation in the exchange activity induced by ATP in the absence of Na\(^+\), and 2) the relief of Na\(^+\) inhibition. B: Na\(^+\)-dependent inhibition of forward Na\(^+\)/Ca\(^{2+}\) exchange at an acid pH of 6.9 in the presence and absence of ATP. C: Na\(^+\)-dependent inhibition of forward Na\(^+\)/Ca\(^{2+}\) exchange at an alkaline pH of 8.8 with and without ATP. Error bars indicate SE. [From DiPolo and Beauché (90).]

![Figure 20](http://physrev.physiology.org/)

**FIG. 20.** Effect of acid and alkaline pH on the Ca\(^{2+}\) affinity of the Ca\(^{2+}\) regulatory site in the stimulation of Na\(^+\)/Na\(^+\) exchange. A: steady-state Na\(^+\)-dependent Na\(^+\) efflux at pH 6.9 induced by increasing the [Ca\(^{2+}\)] from 0 to 200 \(\mu\)M in the presence of 2 mM ATP. Open circles refer to removal of extracellular Na\(^+\). B: percentage Ca\(^{2+}\)-dependent Na\(^+\)/Na\(^+\) exchange at pH 6.9 in the presence and absence of 3 mM ATP. The error bars indicate SE. The mean temperature was 17.5. Notice the large increase in the apparent affinity of the Ca\(^{2+}\)-regulatory site induced by ATP at acidic pH. [From DiPolo and Beauché (90).]
since other nucleotides triphosphate like GTP, UTP, and ITP are ineffective (71). This constitutes a marked difference with other systems, like the Na\(^+/\)H\(^+\)-K\(^+/\)H\(^+\)-ATPase, where CTP, ITP, UTP, and even GTP show variable capabilities in sustaining the overall hydrolysis and also partial reaction cycles (see Ref. 54); however, by themselves, these findings do not rule out an ATPase-related regulation of the exchanger.

**II) Capability to be hydrolyzed.** The hydrolyzable ATP analog 2-deoxy-ATP and AMP-CPP can mimic ATP, while the nonhydrolyzable analogs AMP-PCP and AMP-PNP are ineffective (71, 88). Nonhydrolyzable ATP analogs are also ineffective in mammalian heart under giant patch (118) and mammalian heart and nerve vesicles (26, 27). This rules out an allosteric, nonphosphorylating, role, similar to that reported for the stimulation of the cation transporting ATPases like the Na\(^+/\)K\(^+\)ATPase (20, 206, 240).

**III) Number of phosphates.** Adenosine with either one (AMP) or two (ADP) phosphate(s) is unable to promote stimulation of the exchanger. The same applies for cAMP (71). On the other hand, in the presence of ATP, ADP becomes inhibitory (75).

**IV) Addition of other elements to the phosphate chain.** In adenosine 5\(^\prime\)-O-(3-thiotriphosphate) (ATP\(^{3\prime}\)S), the divalent oxygen of the phosphate in position gamma is replaced with sulfur. The transference of this phosphate gives rise to what is called thio-phosphorylation. In transport ATPases, ATP\(^{3\prime}\)S leads to a reversible thio-phosphorylation of the enzyme in the presence of Mg\(^{2+}\) and the intracellular translocating cation. However, the cycle is halted after the debinding of free ADP, and the enzyme remains in the MgE1S-P form (234). Therefore, ATP\(^{3\prime}\)S is not a substrate for the overall cycle of transport ATPases. With kinases, after thio-phosphorylation of the substrate, the MgADP complex is released. The thio-phosphorylated substrate may lose its thio-phosphate spontaneously and/or by the action of an Mg\(^{2+}\)-activated phosphatase. However, thio-phosphorylation is more stable than phosphorylation from ATP, including a lower sensitivity to

---

**Fig. 21.** Kinetic model for the ATP regulation of the Na\(^+/\)H\(^+\), and Ca\(^{2+}\) interactions with the Ca\(^{2+}\)-regulatory site of the squid Na\(^+/\)Ca\(^{2+}\) exchanger. The broken lines enclosed the intracellular regulatory loop. A–D: steady-state model simulations for the Na\(^+/\), H\(^+/\), Ca\(^{2+}\), and ATP interactions in the modulation of the squid Na\(^+/\)Ca\(^{2+}\) exchanger (see text for details). [From DiPolo and Beaugé (89).] E: pre-steady-state of forward exchange activity at pH\(_i\) 6.9 in the presence and absence of ATP\(^i\). (From L. Beaugé and R. DiPolo, unpublished results.)
phosphatase attack. This means that 1) ATPγS is substrate for kinases, and 2) if a given effect depends on the stability of a kinase-phosphorylated compound, it is likely to be more marked with ATPγS than with ATP, i.e., ATPγS is a better phosphorylase substrate (115). In diazoyl squid axons ATPγS stimulates all exchange modalities provided Mg2+ is present. With ATP, removal of the nucleotide in the presence of Mg2+ leads to a fast and complete deactivation of the exchanger; in the case of ATPγS, after its removal the exchanger is only partially deactivated and at a slower rate (75). In addition, the fractional ATPγS stimulation is consistently greater than that of ATP. This is in line with the greater stability of thio-phosphorylation compared with phosphorylation (57, 149). The major conclusion that can be drawn is that ATP stimulation in squid nerve does not occur via a transport ATPase reaction, but involves one or more kinases. The observation that injection of an unspecific alkaline phosphatase removes MgATP stimulation indicates that the likely target(s) is a protein. On the other hand, a battery of protein kinase inhibitors of PKA, PKC, TyrK, and calmodulin-dependent kinase (see Table 3) were ineffective. Therefore, the responsible kinase(s) must lie outside this group of enzymes. In mammalian cells, the effects of ATPγS are dissimilar, even when the same tissue is analyzed, depending on the experimental preparation. This is intriguing, since, as will be seen below, the same metabolic pathway applies in all these instances. Thus, in giant patches of mammalian heart, the exchange stimulation seen with MgATP is not observed with ATPγS (61). On the other hand, in heart sarclemmal vesicles, ATPγS is as active as MgATP, with an additional intriguing property: it does not require the presence of vanadate (27). Also interestingly, in bovine brain membrane vesicles, although ATPγS does stimulate the exchanger, it is only about one-third of that obtained with MgATP (27).

The β,γ-bidentate chromium-ATP complex is formed by the almost irreversible binding of a chromium atom to the oxidyrl forming oxygen of the β- and γ-phosphate. In transport ATPases, the sequence starts with the chromium-phosphorylation of the enzyme; the cycle is also halted after the debinding of free ADP, and the enzyme remains in the E1Cr-P form (250). Therefore, CrATP is not substrate for the overall ATPase reaction. In many kinases, although not in all, chromium-phosphorylation leads to the formation of the [Cr(H2)4(ADP)(substrate-Pj)] complex. This complex is very stable, and its breakdown is the rate-limiting step of the kinase-CrATP reaction; that rate can be as low as one per minute (102). Consequently, CrATP cannot sustain activity in those kinase systems.

In diazoyl squid axons CrATP has the following effects on Na+/Ca2+ exchange: 1) it does not affect the “basal” exchange fluxes; 2) it does not stimulate the exchanger even at concentrations as high as 3 mM; 3) when added after MgATP has reached its steady-state stimulation, it completely reverts upregulation; and 4) when added before MgATP, it prevents stimulation. Actually, the effect of CrATP is practically irreversible, since MgATP is ineffective more than 1 h after removal of CrATP from the dialysis solution (82). Together with the results obtained with ATPγS, vanadate, inorganic phosphate, pNPP, and protein phosphatases, the effects of CrATP indicate that protein kinase(s) inhibited by this complex is involved in MgATP regulation of the squid nerve Na+/Ca2+ exchanger. Interestingly, CrATP has no effect on PA modulation of the exchanger, which seems also to involve protein phosphorylation (see sect. vB2). So far, there are no studies of CrATP on the exchanger regulation in other systems or preparations. Finally, it must be stressed that other cation exchange systems in squid axons such as Na+/H+ (47) and the Na+/Mg2+ (87) exchanges are also metabolically regulated by kinase reactions. This property of the Na+/Mg2+ exchange has also been described in barnacle muscle fibers (210).

E) Metabolic pathways for ATP stimulation. I) The mammalian heart: role played by phosphatidylinositides. In 1991 and 1995, Luciani and co-workers (166, 167) found that cardiac sarcomemmlal vesicles enriched in their content of phosphatidylinositol 4-phosphate [PtdIns(4)P] and PtdIns(4,5)P2 has an increased Na+/Ca2+ exchange rate. In addition, the deacylation products of polyphosphoinositides acted as powerful inhibitors of the exchanger. That was, indeed, the first experimental evidence that these compounds are related to the exchange function. The next step was the finding that the mechanism of MgATP stimulation was actually through the production of PtdIns(4,5)P2 (125) and involved reversion of the Na+ dependent inactivation. Part of the experimental evidence is described in Figure 22, A–C. The MgATP stimulation of the outward exchange current observed in Figure 22A is absent when the membrane has been previously depleted of the precursor phosphatidylinositol (PtdIns) by the action of a specific PtdIns-PLC (see Fig. 22B); under this condition, addition of PtdIns(4,5)P2 (125) and involved reversion of the Na+ dependent inactivation. Part of the experimental evidence is described in Figure 22A, C. The MgATP stimulation of the outward exchange current observed in Figure 22A is absent when the membrane has been previously depleted of the precursor phosphatidylinositol (PtdIns) by the action of a specific PtdIns-PLC (see Fig. 22B); under this condition, addition of PtdIns(4,5)P2 produces an increment in exchange current similar to that due to MgATP. Furthermore, in a PtdIns-depleted preparation where ATP is ineffective, the inclusion of PtdIns after MgATP leads to stimulation of the exchanger (Fig. 22C). Finally, a specific PtdIns(4,5)P2 antibody eliminated MgATP stimulation (125). Similar results were found in bovine cardiac sarcomemmlal vesicles where the formation of PtdIns(4,5)P2 occurs through a fast phosphorylation cascade; a calcium-independent synthesis of PtdIns(4)P followed by a calcium-dependent production of PtdIns(4,5)P2 (27). This sequence of events is illustrated in the cartoon of Figure 22.

The later developments regarding this subject consisted of the finding that in bovine heart vesicles PtdIns(4,5)P2 and the exchanger protein communoprecipitate. The approach used in these experiments had already been proven successful in studying the binding of
PtdIns(4,5)P₂ to actinin and vinculin, proteins involved in the signaling process by tyrosine kinases (110). The amount of PtdIns(4,5)P₂ that coimmunoprecipitates with NCX1 increases in the presence of MgATP and vanadate (Fig. 23A) and that increase parallels the stimulation of the exchange fluxes (Fig. 23B) (8). In the presence of 1/8 H₂O/MgCl₂, MgATP has no effect either on conditions that affect the MgATP stimulation of the exchanger do so with the coimmunoprecipitated PtdIns(4,5)P₂ (8). 1) With 100 μM Ca²⁺, MgATP has no effect either on

![Diagram](http://physrev.physiology.org/)

**FIG. 22.** Stimulation of cardiac Na⁺/Ca²⁺ exchange current by cytoplasmic ATP and its dependence on phosphatidylinositol (PI) in giant excised inside-out cardiac membrane patches. Horizontal bars indicate the time that a particular substance was applied to the cytoplasmic patch surface. A: outward Na⁺/Ca²⁺ exchange current was activated by replacement of CsCl by NaCl. After current stabilized, ATP (2 mM) activates the outward current. B: patches treated with phosphatidylinositol-specific PLC (PI-PLC) had almost no ATP effect. C: PI-PLC-treated patch recovers their ATP effect by application of PI. [From Hille and Ball (125).] D: cartoon displaying ATP regulation of the cardiac Na⁺/Ca²⁺ exchanger by PI-PLC.

PtdIns(4,5)P₂ bound to bovine heart Na⁺/Ca²⁺ exchanger by Western blot as a function of total membrane protein concentrations. Cardiac sarcolemmal membrane vesicles (10 – 60 μg) were incubated for 20 s at 37°C in the same solutions used for Ca²⁺ uptake experiments. The reaction was stopped by adding Laemmli SDS-sample buffer at five times the final concentration; the samples were then heated for 10 min at 37°C. Discontinuous SDS-PAGE (7.5% acrylamide) was electrotransferred to PVDF membranes for Western blots. In a first step, the blots were incubated with a mouse monoclonal antibody against PtdIns(4,5)P₂ (Per Septive Biosystems) and then developed using a biotinylated streptavidin peroxidase-complex secondary antibody and the Amersham ECL Plus immunoblotting detection system. The bands recognized by specific antibodies were detected and quantified with a Storm 840 image analyzer (Molecular Dynamics) with a Scion PC (NIH) software. In a second step, the same blots were subjected to stripping as indicated by the ECL Plus manufacturer, and then reprobed with a primary guinea pig polyclonal antibody against the NH₂-terminal portion of the bovine cardiac Na⁺/Ca²⁺ exchanger. The effectiveness of the stripping was consistently checked before the second step was started by incubating the PVDF membranes with ECL Plus detection reagents and exposing them to the image analyzer. The band densities obtained from the anti-PtdIns(4,5)P₂ antibody (in arbitrary units) were divided by the image densities obtained from the anti-NCX1 antibody (in arbitrary units). To compare different experiments, a value of 1 was assigned to the ratio of densities obtained in control conditions (presence of 1 μM Ca²⁺ and absence of ATP). The experiments were done in duplicate and repeated at least three times. Note the following: 1) the amounts of PtdIns(4,5)P₂ and Na⁺/Ca²⁺ protein increase in parallel with the increase in total protein, and 2) in the presence of ATP, the levels of PtdIns(4,5)P₂ bound to Na⁺/Ca²⁺ exchanger are markedly increased. B: correspondence of MgATP stimulation of Na⁺/Ca²⁺ exchange fluxes (left) and of the amount of PtdIns(4,5)P₂ bound to exchanger protein (right).

In the case of immunoblots, the PtdIns(4,5)P₂-to-NCX1 ratio with no ATP was arbitrarily given a value of 1. [Redrawn from Asteeggiano et al. (8).]

**FIG. 23.** A: detection of PtdIns(4,5)P₂ bound to bovine heart Na⁺/Ca²⁺ exchanger by Western blot as a function of total membrane protein concentrations. Cardiac sarcolemmal membrane vesicles (10 – 60 μg) were incubated for 20 s at 37°C in the same solutions used for Ca²⁺ uptake experiments. The reaction was stopped by adding Laemmli SDS-sample buffer at five times the final concentration; the samples were then heated for 10 min at 37°C. Discontinuous SDS-PAGE (7.5% acrylamide) was electrotransferred to PVDF membranes for Western blots. In a first step, the blots were incubated with a mouse monoclonal antibody against PtdIns(4,5)P₂ (Per Septive Biosystems) and then developed using a biotinylated streptavidin peroxidase-complex secondary antibody and the Amersham ECL Plus immunoblotting detection system. The bands recognized by specific antibodies were detected and quantified with a Storm 840 image analyzer (Molecular Dynamics) with a Scion PC (NIH) software. In a second step, the same blots were subjected to stripping as indicated by the ECL Plus manufacturer, and then reprobed with a primary guinea pig polyclonal antibody against the NH₂-terminal portion of the bovine cardiac Na⁺/Ca²⁺ exchanger. The effectiveness of the stripping was consistently checked before the second step was started by incubating the PVDF membranes with ECL Plus detection reagents and exposing them to the image analyzer. The band densities obtained from the anti-PtdIns(4,5)P₂ antibody (in arbitrary units) were divided by the image densities obtained from the anti-NCX1 antibody (in arbitrary units). To compare different experiments, a value of 1 was assigned to the ratio of densities obtained in control conditions (presence of 1 μM Ca²⁺ and absence of ATP). The experiments were done in duplicate and repeated at least three times. Note the following: 1) the amounts of PtdIns(4,5)P₂ and Na⁺/Ca²⁺ protein increase in parallel with the increase in total protein, and 2) in the presence of ATP, the levels of PtdIns(4,5)P₂ bound to Na⁺/Ca²⁺ exchanger are markedly increased. B: correspondence of MgATP stimulation of Na⁺/Ca²⁺ exchange fluxes (left) and of the amount of PtdIns(4,5)P₂ bound to exchanger protein (right).

In the case of immunoblots, the PtdIns(4,5)P₂-to-NCX1 ratio with no ATP was arbitrarily given a value of 1. [Redrawn from Asteeggiano et al. (8).]
the PtdIns(4,5)P₂ bound or on the exchange fluxes. 2) XIP inhibits the exchanger. This is more noticeable in the absence of ATP. With XIP, ATP does not modify the levels of bound PtdIns(4,5)P₂ concurrently with a minimal ATP stimulation of the exchanger. 3) Vesicles pretreated with PtdIns-PLC show no de novo [³²P]ATP production of PtdIns(4,5)P₂, although a small ATP-stimulated increase in the bound PtdIns(4,5)P₂ was detected; the levels did not exceed those observed with vanadate and no ATP. A plausible explanation is that, at least in bovine heart sarcolemmal vesicles, ATP upregulation of NCX1 is related to PtdIns(4,5)P₂, but not exceed those observed with vanadate and no ATP. A plausible explanation is that, at least in bovine heart sarcolemmal vesicles, ATP upregulation of NCX1 is related to PtdIns(4,5)P₂ bound to the exchanger over a “threshold” or “unspecific” amount. Also important is the possibility that vanadate influences the amount of detected PtdIns(4,5)P₂ either by inhibiting specific phosphatases and/or by producing a redistribution of PtdIns(4,5)P₂ molecules associated with the NCX1. In summary, ATP up-regulation of the NCX1 in bovine heart sarcolemmal vesicles is related to a newly produced PtdIns(4,5)P₂ in the vicinity of the exchanger and likely bound to it (21).

Another interesting observation in heart sarcolemmal vesicles is that even in the absence of vanadate ATPγS stimulates the exchanger. The de novo production of PtdIns(4,5)P₂ and the bound PtdIns(4,5)P₂ to NCX1 exactly mimics the effects of MgATP (27; V. Posada, L. Beaugé, and G. Berberián, unpublished data). As indicated above, thiophosphorylation is more resistant to hydrolysis than simple phosphorylation. Therefore, vanadate may prevent the action of some phosphatase related to PtdIns(4,5)P₂ hydrolysis. In addition, the conformation of PtdIns(4,5)P₂ and thio-PtdIns 4,5-P₂ must be sufficiently similar to stimulate the exchanger. On the other hand, it is puzzling that mammalian hearts expressing NCX1 show different sensitivity to ATPγS depending on the experimental preparation: little or no effect under excised patch (both in native and heterologous cells expressing the exchanger) (61, 118), and full activation in sarcolemmal vesicles (27; Posada, Beaugé, and Berberián, unpublished data). Incidentally, in bovine brain membrane vesicles, MgATP stimulation is related to PtdIns(4,5)P₂, but ATPγS is no more than one-third as effective. In this case, however, a mixture of exchanger isoforms cannot be ruled out as a possible explanation (26).

Experiments with NCX1 expressed in Xenopus oocytes suggest that PtdIns(4,5)P₂ binds to or near the XIP region of the intracellular loop (116), but the intimate mechanisms of that binding are not known. In general, there are two ways by which PtdIns(4,5)P₂ can bind to a protein; it can bind to specific sites or by strong electrostatic interactions. The NCX1 does not have pleckstrin homology (PH) domains that are the acceptor regions in several proteins that bind PtdIns(4,5)P₂ (108, 172). On the other hand, NCX1 has many arginine residues (172) that could form a positively charged pocket that traps PtdIns(4,5)P₂. However, simple electrostatic interaction may not be enough, since samples treated with up to 2% SDS still show PtdIns(4,5)P₂ bound to the exchanger. The mechanism by which PtdIns(4,5)P₂ activates the exchanger remains unknown, and there is more than one possibility. For example, in cytoskeleton proteins that bind Ca²⁺, regulation by PtdIns(4,5)P₂ leads to PtdIns(4,5)P₂ hydrolysis (110), whereas PtdIns(4,5)P₂ modulation of voltage-dependent ionic channels does not (199).

II) The squid nerve: protein phosphorylation and the need for a cytosolic soluble protein. An initial puzzling observation was that whereas in intact and dialyzed squid giant axons the MgATP stimulated Na⁺/Ca²⁺ exchanger, that effect was not observed in membrane vesicles prepared from squid optic nerve. This coincided with the failure to detect MgATP stimulation in cardiac sarcolemmal vesicles. At that time (P. Baker, personal communication), it was advanced that perhaps a crucial membrane and/or cytosolic component was lost during vesicle preparation. The results described above indicate that this was not the case for mammalian heart. On the other hand, this is what happens with the squid nerve. When squid axons were dialyzed with porous capillaries of high molecular mass cut off (≈18 kDa) for more than 5 h, MgATP-stimulated exchanges fluxes progressively diminished without any effect on the basal fluxes or the Ca²⁺ pump (Fig. 24, A and B) (23, 94), i.e., the ability to exchange Na⁺ and Ca²⁺ was unimpaired since its Vmax remained unchanged but metabolic regulation was lost. This finding led to the idea that a low-molecular-weight cytosolic compound lost during prolonged dialysis was essential, and efforts were started to isolate it. Initially, the starting material was axoplasm extruded from squid giant axons (23); identical results were later obtained by using optic ganglia with the advantage that more material was obtained (94). Figure 25, top, shows the present procedure used for the partial purification of this soluble cytosolic regulatory protein (SCRP). Squid nerve membrane vesicles were used to test for biological activity on the assumption that they did not respond to MgATP because they lack the required cytosolic compound(s); the assumption proved to be correct. All filtrates, except that from a 10-kDa cut-off filter, induced MgATP stimulation of the exchanger. Therefore, it was concluded that the molecular mass was between 10 and 30 kDa. Because heat denaturation and treatment with chymotrypsin rendered the required compound inactive, it was identified as a protein. The 30-kDa filtrate was lyophilized, resuspended, and passed through a Superdex FPLC column. With the use of nerve vesicles, the appearance of MgATP stimulation was observed only in the number 65 elution fraction that corresponded to a molecular mass of ∼13 kDa. When that fraction was injected into axons lacking the MgATP effect due to prolonged dialysis, the upregulation by the nucleotide was restored (23, 94); other fractions were not effective. Similar results were obtained with MgATPγS (Berberián, Di-

Physiol Rev • VOL 86 • JANUARY 2006 • www.prv.org
Polo, and Beaugé, unpublished data) but not with the nonhydrolyzable ATP analogs AMP-PCP and AMP-PNP.

An obvious question was whether the need for SCRP implied a metabolic pathway different from that of the mammalian heart, or was simply required to drive the PtdIns synthesis chain. The experimental evidence indicates that the squid and mammalian metabolic pathways are indeed different: 1) SCRP does not affect the patterns of PtdIns(4)P and PtdIns(4,5)P(2) formation in squid nerve membrane vesicles despite the fact that these patterns are similar to those of mammalian heart (Fig. 26, A and B); 2) in dialyzed squid axons neither the injection of an anti-PtdIns(4,5)P(2) antibody (before or after MgATP addition) nor injection of PtdIns-PLC, at concentrations high enough to reduce the PtdIns levels, have any significant effect on MgATP stimulation of PLC, at concentrations high enough to reduce the PtdIns(4,5)P(2) formation in squid nerve (118). Possible explanations for these results require answers to the question, What is relevant for the metabolic regulation of an exchanger? There appear to be at least three factors: 1) the structure of the exchanger, 2) the biochemical machinery of the cell, and 3) the biochemical environment surrounding the exchanger. The primary structure of the exchanger by itself is not the answer, and the results just described in oocytes confirm that. Answers 2 and 3, alone or in combination, could account for the apparent contradictory results just described. Perhaps, they can even explain why in oocytes expressing Na(+)/Ca(2+) exchangers, MgATP/P(3) did stimulate NCXSQ1 as efficiently as MgATP, whereas it had little or no effect on NCX1 (118).

A potentially important conclusion arises from the analysis of ionic and metabolic regulation of the Na(+)/Ca(2+) exchangers in mammalian heart and squid nerve. The conclusion is that although the metabolic pathways are different, they converge in their final target, that is, to modify the kinetic parameters of the ionic interactions that take place within the large intracellular regulatory loop of the Na(+)/Ca(2+) exchanger.

Finally, the involvement of cytosolic proteins in ion transporter regulation may not be exclusive for the squid Na(+)/Ca(2+) exchanger. The NCX1 mammalian isoform has a PKC-dependent upregulation that does not lead to phosphorylation of the exchanger; to account for these results it was proposed that the signal transmission induced by PKC activators requires a cytosolic molecule (138). Also, a possible role of calcineurin in the cardiac exchanger.

![Diagram](http://physrev.physiology.org/10.220.33.1/30/27/2006/186)
regulation has recently been proposed by Katanosaka et al. (148), who found that the COOH terminus of calcineurin A/H9252, containing the autoinhibitory domain, binds to the beta1 repeat of the central cytoplasmic loop and inhibits the NCX1. The necessity for cytosolic regulatory proteins has also been suggested for the MgATP modulation of the NHE1 sodium/proton exchanger (2).

F) ROLES OF INTRACELLULAR MAGNESIUM. The intra- and extracellular transport sites of the squid Na+/H10001/Ca2+ exchanger highly discriminate between Ca2+ and Mg2+ (10, 75). However, in dialyzed axons Mg2+ inhibit all transport modes even at concentrations within the physiological range (52, 75, 77, 222). A plausible mechanism may involve the Ca2+ regulatory site, since “in vitro” Mg2+ antagonizes the binding of Ca2+ to that site (162).

In the ATP regulation of the squid Na+/Ca2+ exchanger, intracellular Mg2+ plays two substantial, and opposing roles. On the one hand, and this is common to other preparations (27, 118, 124), Mg2+ is essential for ATP stimulation. How much of this is due to Mg2+ by themselves or to the forming of the MgATP complex has not been unambiguously determined. However, as MgATP is the substrate for kinases (102), it is very likely that this is also the case for the Na+/Ca2+ exchanger. On the other hand, in squid axons, Mg2+ promotes deactivation of the ATP upregulated exchanger (93). At a constant ATP concentration of 4 mM, activation of the exchanger reaches its peak at 1 mM Mg2+, declines at higher concentrations, and is nil at 9 mM (K0.5 ~4 mM). This biphasic response to [Mg2+]i is in line with a coupled phosphorylation-dephosphorylation process as the basis for ATP modulation. Actually, if intracellular Mg2+ is removed after ATP activation has taken place, the exchanger remains upregulated for at least 1 h (93). Similar results were obtained with squid nerve membrane vesicles, but in addition, in the vesicles there was no correlation at all between the [Mg2+] related ATP activation and deactivation and the levels of membrane PtdIns(4,5)P2. When explored, both preparations, mammalian heart and squid nerve, showed the same [Ca2+] dependence of PtdIns(4,5)P2 production. (93), thus indicating that phosphoinositides are not related to MgATP modulation in the squid.

The specific process or processes implicated in this Mg2+ activation-deactivation is not known. One possibil-

---

**FIG. 25.** Top: steps in the partial purification of the squid nerve soluble cytosolic regulatory protein (SCRP) required for ATP stimulation of the Na+/Ca2+ exchanger. Bottom: cartoon of the putative regulation of the squid Na+/Ca2+ exchanger by a soluble cytoplasmic regulatory protein (SCRP).
ity is that the SCRP behaves as part of a two component signal transduction system (188); upon arrival of a given signal, it becomes phosphorylated by a membrane-bound kinase and, after playing its modulator role, it loses the phosphate through a Mg$^{2+}$-dependent autophosphatase activity (168; see cartoon at bottom of Fig. 25). Alternatively, other phosphatases, like the protein phosphatase 2C type (186), may come into play.

G) PRELIMINARY BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATIONS OF THE SCRP. Here we provide a summary description of relevant unpublished material from our labora-

![Diagram of phosphoinositides](image)

Fig. 26. Intracellular $^{32}$P labeling from [$\gamma$-$^{32}$P]ATP of phosphoinositides in membrane vesicles from squid optic nerve and bovine heart. A: phosphoimage of 1-dimensional TLC plates. Lanes 1–3 are from nerve membrane vesicles, and lanes 5 and 6 are from bovine heart membrane vesicles. Lane 1, control; lane 2, vesicles with addition of SCRP; lane 3, nerve vesicles treated with phospholipase C (PLC)-specific phosphatidylinositol (PtdIns; 200 U/ml); lane 4, SCRP without nerve membrane vesicles; lanes 5 and 6, control or PtdIns-PLC-treated (20 U/ml) bovine heart membrane vesicles; lane 7, negative control without membranes. Positions of phosphatidylinositol 4-phosphate [PtdIns(4)P; PIP] and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P$_2$; PIP$_2$] are indicated.

![Graph](image)

Fig. 27. Lack of effect of PtdIns(4,5)P$_2$ antibody [Ab-PtdIns(4,5)P$_2$] and PtdIns-PLC on MgATP-activated Na$^+$/Ca$^{2+}$ exchange in dialyzed squid axons. ●, Ca$^{2+}$ efflux in the presence of external Na$^+$; ○, Ca$^{2+}$ efflux in the absence of external Na$^+$. A: intracellular injection of Ab-PtdIns(4,5)P$_2$ during internal dialysis after addition of ATP. B: intracellular injection of Ab-PtdIns(4,5)P$_2$ during internal dialysis before addition of ATP. C: intracellular injection of PtdIns-PLC (final concentration in the axon of $\sim$200 units) before ATP addition. Notice the lack of effect of these compounds on the ATP stimulation of the Na$^+$/Ca$^{2+}$ exchange. [From DiPolo et al. (93).]

G) PRELIMINARY BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATIONS OF THE SCRP. Here we provide a summary description of relevant unpublished material from our labora-
tories (Berberian, DiPolo, and Beaugé, unpublished data). Our initial working hypothesis proposed that the SCRP was a “response regulator” with amino acid composition similar to those described in prokaryotes and some eukaryotes (23). Unfortunately, after spending a lot of time and effort, we were unsuccessful in identifying this factor (M. Delgado, R. DiPolo, and L. Beaugé, unpublished data).

The amounts of SCRP initially obtained when the 30-kDa cut-off filtrate was passed through a FPLC Superdex-75 column was minimal. An alternative approach was to purify further the 30-kDa cytosolic fractions. When that fraction went through anionic (Dowex 1 × 8 – 400) and cationic (Dowex 50 × 8 – 400) exchange columns, biological activity was recovered only in the anionic column, indicating a net positive charge of the SCRP. Furthermore, the eluent of an anionic column retained by a 10-kDa cut-off filter was used for tests (10- to 30-kDa fraction; Fig. 25). Coomassie blue-stained gels showed several bands, two of them quite conspicuous, ~25 and 13 kDa. This fraction was then exposed to 0.5 mM [32P]ATP in the same solution used for transport assays in squid optic nerve membrane vesicles under a variety of conditions. Only in the presence of native membrane vesicles did the cytosolic fraction become phosphorylated, and phosphorylation occurred in the two major bands, one around 25 kDa and another, more prominent, around 13 kDa. This means that 1) this 10- to 30-kDa fraction has no kinase activity and 2) the kinase(s) responsible for its phosphorylation is located in the plasma membrane of the cells. Other important findings include 1) membrane vesicles alone do not show phosphorylated bands around 10–30 kDa and 2) heat denaturation of the cytosolic fraction of the membrane vesicles prevented phosphorylation. This agrees nicely with the observation that the heat-denatured fraction is unable to promote MgATP stimulation of the exchanger (23, 94).

Staurosporine, particularly at high concentrations, is a general inhibitor of kinases (212). In dialyzed axons, MgATP stimulation of the Na⁺/Ca²⁺ exchanger is insensitive to a large variety of kinases and phosphatase inhibitors, including staurosporine (94). In squid nerve vesicles, staurosporine (up to 50 μM) does not affect MgATP + SCRP stimulation or the phosphorylation of the 13-kDa cytosolic bands. At the same time, most of the phosphorylated bands from the membrane vesicles disappear. This has two important implications: 1) it supports the idea that the 13-kDa band is involved in MgATP stimulation, and 2) staurosporine eliminates a considerable number of unrelated kinases, becoming an excellent tool for further analysis of protein phosphorylation related to stimulation of the exchanger.

The SCRP phosphorylated bands were sent for amino acid sequencing (W. M. Keck Biomedical Mass Spectrometry Laboratory, Univ. of Virginia Health System). Analysis of peptides from the 25-kDa band allowed the identification of two known proteins: Calexcitin B and RAB2. The peptide from the 13-kDa band did not correspond to any known protein and is considered a de novo species. Previous results (23, 94) suggest that the 13-kDa protein is the most likely candidate for the SCRP. RAB2 does not seem relevant, since it is a GTP-binding protein associated with Ras that regulates vesicular transport from ER to Golgi membranes and shows GTPase activity (235). This does not appear compatible with the nucleotide regulation of the squid Na⁺/Ca²⁺ exchanger where GTP and GTP·γS are ineffective (88, 89). The 25-kDa band (although in some cases the phosphorylation of this band is minimal) will require additional studies. Calexcitin B (CEB) is a neural calcium sensor protein that is phosphorylated by

![FIG. 28. Effects of PtdIns(4,5)P₂ incorporation into membrane vesicles on the Na⁺ gradient-dependent []Ca²⁺ uptake in squid nerves (A) and bovine heart (B). Vesicles were preincubated for 5 min at 0°C and an additional minute at 20°C with PtdIns(4,5)P₂ liposomes (0.25 mg/mg vesicles) protein vesicle. []Ca²⁺ uptake was measured for 15 s at 20°C. Notice the following: 1) the usual MgATP stimulation is observed in both preparations, and 2) the complete lack of effect of PtdIns(4,5)P₂ in the squid Na⁺/Ca²⁺ exchange fluxes in contrast to the marked stimulation in the heart. All values are means ± SE of quadruplicate determinations. [From DiPolo et al. (93).]
PKC and does not bind GTP. CEq is expressed in the *L. pedet* optic lobe and is considered to be a new member of the family of sarcoplasmic calcium-binding proteins (114).

Finally, we found that the phosphorylated factor could stimulate the \( \text{Na}^+/\text{Ca}^{2+} \) exchanger in squid nerve vesicles even in the absence of ATP. Native SCRP was compared with SCRP phosphorylated from MgATP phosphorylated SCRP in the presence of membrane vesicles. Phosphorylation was stopped by adding EDTA, and the vesicles were separated by centrifugation in an Airfuge. The phosphorylated SCRP of the supernatant stimulated \( \text{Na}^+/\text{Ca}^{2+} \) exchange fluxes in a manner similar to that seen with native SCRP + MgATP. Interestingly, as happens with ATP, stimulation by the phosphorylated SCRP did not take place in the absence of millimolar \([\text{Mg}^{2+}].\) Therefore, one can conclude the following. 1) The 10- to 30-kDa fraction not only contains the essential cytosolic factor(s), but that to exert its action, this factor must be phosphorylated. 2) Phosphorylation of the 10- to 30-kDa fraction requires at least one MgATP-dependent protein phosphorylating step. 3) Regardless of the specific biochemical reaction(s) by which the 10- to 30-kDa fraction stimulates the exchanger, either by the transfer of its phosphate to another structure, or just by binding to a target receptor, \([\text{Mg}^{2+}] \) is required but MgATP is no longer needed.

**H) PHOSPHORYLATION OF THE SODIUM/CALCIUM EXCHANGER PROTEIN.** We investigated whether the \( \text{Na}^+/\text{Ca}^{2+} \) exchanger protein became phosphorylated under the condition in which MgATP stimulates exchange fluxes in both squid nerve membrane (NCXSQ1) and bovine heart sarcolemmal (NCX1) vesicles. Numerous phosphorylated membrane proteins were resolved in a 8% SDS-PAGE, including proteins at about the molecular weight corresponding to the exchangers, but neither NCX1 nor NCXSQ1 immunoprecipitated with a specific antibody showed any \([\text{32P}]\) incorporation. In addition, in the squid, the phosphorylation pattern of the total membrane proteins is the same with or without the SCRP (Berberián, DiPolo, and Beaugé, unpublished data). In other preparations the results are variable. No phosphorylation was observed in the immunoprecipitated ATP-stimulated bovine cardiac \( \text{Na}^+/\text{Ca}^{2+} \) exchanger transfected in CHO cells (63).

In contrast, phosphorylation of NCX1, related to PKC and PKA, has been observed in canine exchanger expressed in transfected cells, isolated rat cardiac myocytes in culture (142), and cardiac and kidney NCX1 isoforms expressed in *Xenopus* oocytes (226, 227). These phosphorylations were associated with an increase in the activity of the exchanger, which was much smaller that those seen in squid nerve, giant cardiac patches, or cardiac sarcolemmal vesicles. Furthermore, their relationships with ions interacting with the intracellular loop were not established.

1) **USE OF INHIBITORS AS TOOLS TO UNDERSTAND IONIC AND ATP MODULATION OF THE SODIUM/CALCIUM EXCHANGER.** In recent years a series of organic compounds that inhibit \( \text{Na}^+/\text{Ca}^{2+} \) exchange have been synthesized. Some of them seem to be particularly relevant for the study of ionic and ATP regulation of the exchanger. For instance, it has been suggested that 2-[2-[4-(4-nitrobenzoxoxy)phenyl]ethyl]isothio urea (KB-R7943) and the more potent 2-[4-[2,5-dihurophenyl]methoxy]phenoxyl]-5-ethoxyanline (SEA4004) (Fig. 29, A and B) reduce the reverse exchange mode (\( \text{Na}^+ \)-dependent \( \text{Ca}^{2+} \) influx) by acting from the extracellular surface (134, 135, 143, 160, 244, 253). Amino acid mapping indicates that Gly 833, on the \( \alpha-2 \) repeat region, is critically important for inhibition (136). In fibroblasts expressing NCX1 and excised membrane patches of *Xenopus* oocytes expressing NCX1.1, SEA favors the transition of the exchangers into the \( \text{Na}^+ \)-dependent inactive state; expressed exchangers with the XIP region either mutated or deleted become resistant to the inhibitor (48). Another newly synthesized compound, 2-[4-(-4-nitrobenzoxoxy)-benzyl]thiazolidine-4-carboxylic acid ethyl ester (SN-6), also inhibits the reversal exchange in a manner that required the integrity of the XIP region, suggesting that its effect is related to \( \text{Na}^+ \) inactivation (134). In these cases NCX1 was more sensitive than NCX2 and NCX3. An important additional finding with SN-6 was that ATP depletion enhanced inhibition (see Fig. 29A) (134). In dialyzed squid axons SEA inhibits all transport modes of the exchanger from both membrane sides, but its potency is increased when applied internally (19). Other characteristics of SEA inhibition included 1) increase of the synergic (\( \text{Na}^+ + \text{H}^+ \)) inactivation, 2) enhancement by intracellular acidification even in the absence of \( \text{Na}^+ \), and 3) its prevention by MgATP and by intracellular alkalization (see Fig. 30). These findings can be explained by the model recently proposed for the squid \( \text{Na}^+/\text{Ca}^{2+} \) exchange regulation (90). According to that scheme SEA would act on the (\( \text{H}^+ + \text{Na}^+ \))-inactivation process by increasing the affinity of \( \text{H}^+ \) and \( \text{Na}^+ \) binding to their inhibitory sites. Protection by ATP would come from its ability to antagonize (\( \text{H}^+ + \text{Na}^+ \)) synergetic inhibition by reducing the binding affinities for \( \text{H}^+ \) and \( \text{Na}^+ \); protection by alkalinization would occur because protonation of the carrier is required for \( \text{Na}^+ \) to get attached to its inhibiting site (19). An additional important point supporting this hypothesis is that in the squid the SEA effects are poorly reversible or nonreversible, whereas the \( \text{Na}^+ + \text{H}^+ \) inhibition enhanced by SEA is fully reversible (19, 90).

2. **The squid nerve: modulation of the \( \text{Na}^+/\text{Ca}^{2+} \) exchanger by phosphoryriene**

Several biologically active molecules act through what is called a “group transfer potential”, chemical groups that, when transferred to another molecule, lead
to a change in the chemical potential (or free energy). Acetyl, methyl, or phosphate groups can play this role. Actually, ATP "releases" energy from the transfer of its gamma phosphate. In addition to ATP, other molecules include 1) those with transfer potentials lower than ATP (in descending order): phosphoarginine; acetyl-S-esters, glycyglycine, glutamine, glucose 6-phosphate, glycerol 1-phosphate; and 2) those with transfer potentials higher than ATP (in ascending order): acetyl-phosphate, creatine phosphate, enol pyruvate phosphate, and acetyl imidazol. The usual ATP concentration in normal cells is in the millimolar range (1–4 mM). Phosphoarginine (PA), in mollusks and other invertebrates, and phosphocreatine (PCr), in vertebrates, are also present in millimolar amounts in the cytosol. These last two compounds differ only in a methyl group; their group transfer potential is quite different though and is related to a phosphate attached to a guanidine residue. The main function of PA and PCr is to buffer [ATP] by phosphorylating ADP through the action of arginine and creatine kinases.

A nonnucleotide potential group transfer molecule like acetyl phosphate can act as substrate for the Na\(^+\)-K\(^+\)-ATPase (or Na\(^+\) pump). It can replace, and competes with, ATP in its phosphorylating role, leading to the same phosphointermediates, but it does not do so in the ATP regulatory site (17, 22, 249). On the other hand, PA does not fuel the Na\(^+\)-K\(^+\) pump in the squid (51). In squid axons, acetyl phosphate does not affect the Na\(^+\)/Ca\(^{2+}\) exchanger (DiPolo and Beaugé, unpublished data), whereas PA is a potent upregulator. This effect is fully reversible with a K\(_i\) of ~7–8 mM, close to the normal 10 mM PA concentration in the axoplasm. The experimental evidence to be reviewed below shows that PA does not simply replace ATP, but it has a role of its own; it acts independently and through a metabolic pathway different from, and in parallel with, that of the nucleotide. In addition, the kinetics consequences are also different (85, 87, 90).

Figure 31A describes the stimulation by 5 mM PA of the forward Na\(^+\)/Ca\(^{2+}\) exchange (almost 5-fold in this case) in a dialyzed squid axon. Upon removal of external Na\(^+\), the efflux of Ca\(^{2+}\) drops to leak values, indicating that PA does not stimulate any other Ca\(^{2+}\) extruding
systems, i.e., it does not affect the Ca\(^{2+}\) pump. At the end of the experiment the measurement of ATP concentration in the dialysis effluent gave values below 1 \(\mu\)M, showing that no ATP regeneration occurred after it was washed out of the axoplasm; in other words, ADP was also effectively removed by dialysis. Another important observation shown in Figure 31B is that neither 5 mM arginine nor 5 mM PCr reproduces the stimulation seen with 5 mM PA.

In the case of arginine, the results were negative with and without the simultaneous presence of 5 mM Pi (data not shown). Furthermore, and in contrast with ATP, Figure 31C shows that PA stimulation does not require a cytosolic factor: running down of the ATP effect by prolonged dialysis does not prevent PA stimulation (notice that the MgATP stimulation of the Ca\(^{2+}\) pump is unchanged). An analogous PA stimulation of the exchanger is obtained in squid nerve membrane vesicles with and without the addition of the SCRP (see below). Taking aside the kinetic consequences, other differences between the phosphagen and the nucleotide are as follows: 1) while CrATP completely blocks the MgATP effect, it does not influence PA activation; and 2) neither vanadate nor the phosphatase substrate \(p\)-nitrophenylphosphate, which stimulate the exchanger in the presence of MgATP, influences the exchange fluxes in the presence of PA alone. In addition, the absolute PA stimulation is the same in the absence and in the presence of saturating concentrations of ATP, i.e., the PA and ATP effects are additive. On the other hand, MgATP and PA upregulations have two things in common: 1) both are insensitive to a series of phosphatase (okadaic acid, microcystin) and kinase (staurosporine, H-7, tyrophostin, genistein, lavendustin) inhibitors, and 2) their effects are fully inhibited by unspecific alkaline phosphatases. The later observations indicate that both processes are mediated by protein phosphorylation.

A) KINETIC EFFECTS OF PA MODULATION. Unlike MgATP, PA does not modify the apparent affinities of the external transport sites for Na\(^+\) or Ca\(^{2+}\) or the affinity for the external monovalent activating cations. MgATP stimulation is similar in all modes of translocation (forward, reverse, Na\(^+/Na_0\)\(^-\), and Ca\(^{2+}/Ca_0\)\(^2+\) exchanges); on the contrary, PA activates, preferentially, the modes associated with Ca\(^{2+}\) exit, that is, forward and Ca\(^{2+}/Ca_0\)\(^2+\) exchange, whereas little effect is seen in the transport modes asso-

---

**Fig. 31.** A: effect of phosphoarginine (PA) on Ca\(^{2+}\) efflux in the virtual absence of ATP. All concentrations are in millimolar (mM) unless specified. ○, Ca\(^{2+}\) efflux in the presence of Na\(^+\) and Ca\(^{2+}\); ○, Ca\(^{2+}\) efflux in the absence of Na\(^+\) and Ca\(^{2+}\). The [ATP] in the axoplasm at the end of the experiment was <1 \(\mu\)M. The arrows indicate the addition of 5 mM PA. Notice the lack of effect of PA on the Ca\(^{2+}\) pump and hence the absence of ATP production from ADP. [From DiPolo and Beaugé (85).] B: lack of effect of arginine and phosphocreatine of the squid Na\(^+/Na_0\)\(^-\)-dependent Ca\(^{2+}\) exchanger. Notice the large activation induced by PA on the Na\(^+\)-dependent Ca\(^{2+}\) efflux (forward exchange) (DiPolo and Beaugé, unpublished results). ○, effect of prolonged dialysis on the ATP and PA stimulated Na\(^+\)-dependent Ca\(^{2+}\) efflux. All concentrations are given as millimolar. The arrows indicate the removal or addition of compounds to the dialysis medium. ○, Ca\(^{2+}\) efflux in the presence of Na\(^+\); ○, Ca\(^{2+}\) efflux in the absence of Na\(^+\). This axon was dialyzed with a standard dialysis medium containing no ATP over 4.3 h prior to the addition of 2 mM ATP. Note the absence of ATP-dependent, Na\(^+\)-dependent Ca\(^{2+}\) efflux. Only the Ca\(^{2+}\) pump remains and is inhibited by the addition of 100 \(\mu\)M vanadate (Van). Note that addition of 5 mM PA (after 5.7 h) causes its usual activation of the Na\(^+\)-dependent Ca\(^{2+}\) efflux. [From DiPolo and Beaugé (87).]
associated with Na\(^+\) extrusion (reverse and Na\(^+\)/Na\(_0\)\(^+\) exchange). In addition, as shown in Figure 32, the forward exchange is stimulated much more than the Ca\(^2+\)/Ca\(_0\)\(^2+\) exchange. However, this is due to an overall rate-limiting step related to Ca\(^2+\) entry that is independent of the mechanism by which the exchanger is stimulated; for instance, it is also seen when the exchanger is stimulated by internal alkalinization (75). The relevant effect of PA and what makes a substantial difference with MgATP is that PA has no effect on the Na\(^+\)/Na\(_0\)\(^+\) and Ca\(^2+\)/Ca\(_0\)\(^2+\) interactions with the intracellular “regulatory” loop (Fig. 33A). The main, and so far the only, action of PA is to increase the affinity of the intracellular transport sites, preferentially, for Ca\(^2+\) (~20-fold) over Na\(^+\) (only 50%; not shown). Figure 33B depicts the Na\(_0\)\(^+\)-dependent Ca\(^2+\) efflux as a function of [Ca\(^2+\)]\(_i\) at a pH\(_i\) of 8.8 and starting at 5 \(\mu\)M [Ca\(^2+\)]\(_i\); that is, with the Ca\(^2+\) regulatory site fully saturated (89). Notice that the \(K_{0.5}\) for Ca\(^2+\) stimulation of the forward exchange goes from 200 \(\mu\)M without to 10 \(\mu\)M [Ca\(^2+\)]\(_i\) with PA, i.e., there is a 20-fold increase in the apparent affinity of the intracellular transport sites for Ca\(^2+\). This finding explains why PA favors the forward over the other exchange modes. In addition, PA should make Ca\(^2+\) a more effective competitor with Na\(^+\) for these sites. This was verified in experiments like that described in Figure 33C where the reverse exchange (Na\(^+\) exiting in exchange for Ca\(_0\)\(^2+\)) is more sensitive to Ca\(_0\)\(^2+\) inhibition in the presence than in the absence of PA.

Further evidence that PA effects are unrelated to the ionic and ATP modulation of exchanger comes from experiments with squid nerve membrane vesicles under controlled chymotrypsin digestion (Fig. 34A). In control vesicles (Fig. 34A, left), ATP plus SCRP induces the usual increase in exchange fluxes (23, 93, 94). Without the SCRP, PA induces an even larger stimulation and the combination of ATP plus SCRP plus PA takes the fluxes to a level equal to the sum of (ATP + SCRP) plus PA alone; again, PA and ATP effects are additive (87). In chymotrypsin-treated vesicles (Fig. 34A, right) the basal Na\(^+\) gradient-dependent \(^{45}\)Ca uptake doubles and becomes insensitive to ATP plus SCRP, i.e., the exchanger is deregulated (121, 127). Nonetheless, the exchange fluxes still responded to PA in a manner similar to that seen in the controls.

Figure 34B shows that chymotrypsin affects the integrity of the exchanger, including that of the large regulatory loop; this can be detected with antibodies against the NH\(_2\) terminal portion of the exchanger protein and against the loop. It could be argued that although the loop is indeed disrupted, there is no way to know how much is deleted, and how much remains attached to the main protein, and PA may act precisely on the region of the loop that remains attached to the main protein. Indeed,
we do not know exactly where PA acts. The experimental evidence shows, unambiguously, that PA increases the affinities of the intracellular cation transport sites, mainly for Ca\(^{2+}\), without affecting the maximal translocation rate, and independent of the H\(^{+}\)-Na\(^{+}\)-Ca\(^{2+}\) interactions. If, as proposed, the transport sites are at or near the alpha repeats associated with the ionophoric regions (195, 204), they are likely far from the main intracellular loop. As PA regulation remains intact after chymotrypsin digestion, it is not unreasonable to consider that its overall effects occur at a site distant (or distinct) from the regulatory loop, which is a profound topological difference from that of ATP regulation. However, this does not eliminate the possibility that the loop might in some way be involved in PA regulation. In any event, the chymotrypsin data demonstrate that PA does not share these regions with ATP.

Another interesting property of the squid clone expressed in frog oocytes is its insensitivity to PA (118). This PA insensitivity could result from a lack of a phosphagen regulatory mechanism in the clone, a biochemical machinery of the cell unable to handle PA and/or to the difference in the biochemical environment surrounding the exchanger.

**C) PRELIMINARY BIOCHEMICAL EXPERIMENTS ON PA REGULATION: THE SQUID SODIUM/CALCIUM EXCHANGER.** We began to explore

---

**Fig. 33.** Kinetic parameters of the squid Na\(^{+}/\)Ca\(^{2+}\) reaction cycle modified by PA. **A:** lack of effect on the H\(^{+}\)-Na\(^{+}\) inhibitory synergism. **B:** marked increased in the affinity of the Ca\(^{2+}\) transport site by PA. **C:** increase in the affinity of Ca\(^{2+}\) by PA enhances inhibition of the Na\(^{+}\) transport site. [From DiPolo et al. (91)].
protein phosphorylation in squid nerve membrane vesicles at millimolar concentrations of [32P]PA (Berbería, DiPolo, and Beaugé, unpublished data); to that end, we used a fully competent synthesized [32P]PA, free of contaminants with specific activity of 1–2 × 10^3 cpm/pmol (24). The first observation was that the phosphorylation patterns of ATP and PA are different. Two bands phosphorylated by PA, at 60 and 70 kDa, are not seen with MgATP; conversely, the ATP phosphorylated bands do not show with PA. Amino acid sequencing by mass spectrometry (W. M. Keck Biomedical Mass Spectrometry Laboratory, Univ. of Virginia) of the PA bands reveals a mix of proteins: the major band corresponds to a low-molecular-mass (60–70 kDa) neurofilament (small NF); the others, in much lower quantity, are tubulins A and B. The small NF and the Na+/Ca2+ exchanger proteins were verified in Western blots with specific antibodies (kindly provided by Dr. H. C. Pant and Dr. K. Philipson, respectively). Interestingly, while MgATP does not phosphorylate the 60–70 NF, it does phosphorylate the 200- to 220-kDa large neurofilament (144); conversely, PA does not phosphorylate the high-molecular-mass NF. So far, we have found no data on the properties of the small-molecular-mass NF other than in the squid it is associated with plasma membrane and cytosolic structures. An additional observation was that the PA-phosphorylated NF is quite labile, losing the [32P]Pi upon immunoprecipitation.

Three other aspects of the PA phosphorylation of the small NF were investigated: 1) specificity, 2) stability, and 3) staurosporine.

With regard to specificity, phosphorylation is specific for PA because it is attenuated by cold PA but not by ATP, AMP-PCP, or Pi.
With regard to stability, the phosphorylated 60–70 kDa NF is acid-stable, labile at alkaline pH, and even at pH 7.6, and insensitive to hydroxylamine. These characteristics are consistent with phosphoserine and/or phosphothreonine residues (182). Actually, if PA does phosphorylate the exchanger, and P\textsubscript{i} incorporation is as labile as that of the 60–70 kDa NF, it is still possible that it was missed during its immunoprecipitation.

With regard to staurosporine, PA phosphorylation of the NF is not affected by staurosporine even at concentrations as high as 100 \( \mu \text{M} \). This result is in agreement with the staurosporine insensitivity of PA stimulation of

\[ \text{FIG. 35. Integrated kinetic model for ATP and PA modulation of ionic interaction with the squid Na}/\text{Ca}^{2+} \text{exchanger. Broken lines separate two regions: the regulatory intracellular loop and the transmembrane transport sites. A: lack of effect of PA on the H}/\text{Na}^{+} \text{synergic inhibition. B and C: increase in the affinity of the intracellular Ca}^{2+} \text{transport sites due to PA. Note that in this case the Ca}^{2+} \text{regulatory site is saturated at pH} \text{ of 8.8 at all Ca}^{2+} \text{concentrations. [Modified from DiPolo et al. (91).]} \]
the exchanger in dialyzed squid axons (87) and squid nerve membrane vesicles (data not shown).

At this stage, we do not know if the small NF indeed plays a role in the PA stimulation of the exchanger; a great deal of work has to be done to elucidate that question. However, the fact that PA is able by itself to phosphorylate a protein related to membrane and cytoskeleton structures opens a relevant field for research on the metabolic function of this phosphagen, which may go beyond the regulation of the Na+/Ca2+ exchanger.

VII. SUMMARY: CONCLUSIONS ON THE RELATIVE EFFICIENCY OF THE CALCIUM EXTRUSION MECHANISMS IN SQUID AXONS

Two Ca2+ extrusion mechanisms work in parallel in the plasma membrane of squid nerve cells: a high-affinity, low-capacity Na+/Ca2+ pump and a low-affinity, high-capacity Na+/Ca2+ exchanger (80). The Ca2+ pump has a Km for Ca2+ of ~0.2 μM and a maximal rate of extrusion of ~250 fmol · cm−2 · s−1 (73); as with all cation pumps, it does not work without MgATP. On the other hand, whereas the Na+/Ca2+ exchange can function without MgATP and PA, both compounds markedly influence its activity. MgATP acts by antagonizing the Hi−-Na+ synergistic inhibition and increasing the apparent affinity of the Ca2+ regulatory site, without affecting the transport site’s affinities for Ca2+ or Na+ or the maximal translocation rate.

Conversely, PA does not influence the ionic interactions with the Ca2+ regulatory site, but does increase, ~20-fold, the affinity of the intracellular Ca2+ transport sites with only a slight increase (no more than 50%) of the affinity of those sites for Na+. As with MgATP, PA does not affect the maximal rate of translocation. The basal affinity observed for transporting sites was ~200 μM for Ca2+ and 50 mM for Na+. The resting [Ca2+]i in the squid nerve is about, or less than, 100 nM. During synaptic transmission, the peak concentration in microdomains of the synaptic region may be as high 300 μM (239). Therefore, it is likely that [Ca2+]i near the exchanger is also high, particularly if this mechanism is near the microdomains just mentioned.

As a final step of this review, it seemed interesting to explore the relevance of the Ca2+ pump and the Na+/Ca2+ exchanger in the extrusion of Ca2+ as a function of [Ca2+]i. To that purpose we performed simulations based on squid data (Fig. 36). The concentrations of Ca2+ are indicated on Figure 36; other intracellular concentrations relevant to the simulations were 40 mM Na+, pH 7.3, none or 3 mM ATP, and none or 10 mM PA. Figure 36A is a simulation of the forward exchange from 0 to 10 μM Ca2+ plotted in a linear scale, and Figure 36B is a semi-logarithmic plot from 1 to 300 μM Ca2+. Figure 36A shows, distinctly, the dominant role of the Ca2+ pump at concentrations of cytosolic Ca2+ below 1 μM. Above 1 μM Ca2+ the role of the Na+/Ca2+ exchanger becomes more and more prominent as the concentration of Ca2+ increases. It is interesting to consider what to expect in axons without and with the two high-energy metabolic compounds. In the absence of both ATP and PA, 10 μM Ca2+ is needed in order for the exchanger to match the Ca2+ pump. In the presence of ATP alone, although the exchanger works better, still it only surpasses the pump activity above 7 μM Ca2+, and at 10 μM is just 50% more productive. On the other hand, the simultaneous presence of ATP and PA results in an extremely active exchanger that at concentrations of Ca2+ as low as 2 μM is twice as efficient as the Ca2+ pump. The importance of PA regulation of the Na+/Ca2+ exchanger is even more dramatically shown in Figure 36B. Above 50 μM Ca2+ the pumped Ca2+ efflux is just a minor component of the total efflux, but, more important, the PA-stimulated exchanger dominates the scene,
and it does in such a way that ATP modulation is almost irrelevant. Actually, the PA regulated Na⁺/Ca²⁺ exchanger is so efficient in extruding Ca²⁺ that it can operationally be considered a genuine Ca²⁺ pump.

ACKNOWLEDGMENTS

We thank Hector Rojas, Magaly Ramos, Daniel Delgado (IVIC), and Myriam Siravegna (Instituto M. and M. Ferreyra) for technical support and Jorge Rivas (Photography Department, IVIC) for the art work. We also thank Fundación Polar (Venezuela) and Fundación Interior Argentina for their constant support to science. We are very grateful to Profs. Mordecai Blaustein, Donald Hilgemann, Kenneth Philipson, and John Reeves for their critical revision and suggestions on the manuscript.

Addresses for reprint requests and other correspondence:
R. DiPolo, Laboratorio de Pernenebilidad Ionica, Centro de Biofisica y Bioquimica, IVIC, Apartado 1827, Caracas 1020A, Venezuela (e-mail: dipolor@ivic.ve); L. Beauge, Laboratorio de Biofisica, Instituto de Investigacion Medica “Mercedes y Martin Ferreyra,” Casilla de Correo 389, 5000 Cordoba, Argentina (e-mail: lbeauge@immf.uncor.edu).

GRANTS

This work was supported by United States Science Foundation Grant MCB 0444598, Fonacit Grants SL-990000946 and G-2001000637 (Venezuela), and Foncyt Grant PICT-05–12397 (Argentina).

REFERENCES


Hilgemann DW and Ball R. Regulation of cardiac Na+/Ca2+ exchange and MgATP potassium channels by PIP2. Science 273: 956–960, 1996.


METABOLIC AND IONIC REGULATION OF Na+/Ca2+ EXCHANGE


225. Ringer S. A further contribution regarding the influence of the different constituents of the blood on the contraction of the heart. *J Physiol* 4: 29–42, 1883.


