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Na^+/Ca^{2+} ANTIPORT IN THE MAMMALIAN HEART

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Summary

The cardiac Na^+/Ca^{2+} antiporter moves 3 Na^+ across the plasma membrane in exchange for a single Ca^{2+} moving in the opposite direction. It is the principal Ca^{2+} efflux mechanism in myocardial cells; however, it also contributes to Ca^{2+} influx under certain conditions. It is particularly abundant in the heart, but is also expressed in other tissues such as smooth and skeletal muscle, the kidney and the brain. The cardiac antiporter itself is a protein of 938 amino acids, with a cleaved NH_2-terminal signal sequence, 11 putative transmembrane segments and a large hydrophilic domain of 520 amino acids between the fifth and sixth transmembrane segments. Alternative mRNA splicing mechanisms generate tissue-specific isoforms in a limited region within the hydrophilic domain. Most of the hydrophilic domain can be deleted without altering the kinetics of the transport reaction; the regulatory properties of the antiporter are markedly affected by this deletion however. Two different modes of regulation of antiport activity have been characterized and appear to involve two different inactive states of the carrier. The first is promoted by the presence of cytosolic Na^+ in the absence of ATP and the second is promoted by the absence of cytosolic Ca^{2+}. ATP-dependent regulation of antiport activity may involve interactions with the cellular cytoskeleton, since the effects of ATP depletion can be mimicked by cytochalasin D. Ca^{2+}-dependent regulation of antiport activity appears to involve the interaction of cytosolic Ca^{2+} with two acidic amino acid sequences within a limited region of the hydrophilic domain.

Introduction

Na^+/Ca^{2+} antiporters are found in many, but not all, types of cells (see Blaustein *et al.* 1991). In general, they move Ca^{2+} in one direction across the plasma membrane in exchange for 3 or 4 Na^+ moving in the opposite direction. The exchange reaction is thought to involve a consecutive reaction mechanism, in which Na^+ and Ca^{2+} are translocated in separate steps (Hilgemann *et al.* 1991). Because of the charge imbalance, Na^+/Ca^{2+} antiporters generate a current during their operation and these currents provide an important experimental tool for investigating their activity. There are two general types of plasma membrane Na^+/Ca^{2+} antiporters. The cardiac type, which will be the focus of the present article, has a stoichiometry of $3Na^+/1Ca^{2+}$ (Reeves and Hale, 1984;

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Kimura *et al.* 1987). This antiporter is particularly abundant in the heart, but is also found in brain, kidney, smooth muscle, skeletal muscle and a variety of secretory cells. The second type of antiporter is found in the retinal rod. Ca^{2+} is cotransported with K^+ by this antiporter and the stoichiometry is $4Na^+/(1Ca^{2+}+1K^+)$ (Schnetkamp *et al.* 1989; Cervetto *et al.* 1989). The distribution of the retinal rod antiporter is very limited in comparison with that of the cardiac type. It has been definitively identified only in the retinal rod, although a recent report describes a K^+-dependent Na^+/Ca^{2+} antiporter in human platelets (Kimura *et al.* 1993). Both antiporters have been cloned, but there is surprisingly little amino acid homology between them (see below). There is also a Na^+/Ca^{2+} antiporter, which catalyzes Ca^{2+} efflux from the mitochondria. This antiporter appears to be unrelated to the plasma membrane antiporters, but it has not been very well characterized and will not be discussed further in this chapter.

The major physiological function of the Na^+/Ca^{2+} antiporter is to transport Ca^{2+} out of the cell. In the heart, the antiporter is the principal efflux mechanism for cellular Ca^{2+} (Hilgemann, 1986; Bers and Bridge, 1989) and competes with the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase for cytosolic Ca^{2+} (Fig. 1). Approximately 20 % of the Ca^{2+} released by the SR is transported out of the cell *via* the Na^+/Ca^{2+} antiporter with each beat, and most of the remainder is re-accumulated by the SR. Changes in the driving force for Na^+/Ca^{2+} antiport activity therefore exert a profound influence on the amount of Ca^{2+} taken up by the SR and on the force of contraction of subsequent beats (Fig. 1).

The thermodynamic driving force for Na^+/Ca^{2+} antiport can be expressed in terms of its reversal potential, i.e. the membrane potential at which the exchange system is in equilibrium:

$$\begin{aligned} E_{Na/Ca} &= 3E_{Na} - 2E_{Ca} \\ &= -\boldsymbol{R}TF^{-1}\ln\{([Ca^{2+}]_o/[Ca^{2+}]_i)([Na^+]_i/[Na^+]_o)^3\}, \end{aligned} \quad (1)$$

where $E_{Na/Ca}$ is the reversal potential, E_{Na} and E_{Ca} are the equilibrium potentials for Na^+ and Ca^{2+} as defined by the Nernst equation, $\boldsymbol{R}$ is the gas constant, T is the absolute temperature and F is Faraday's constant. At membrane potentials that are more negative than $E_{Na/Ca}$, current will flow into the cell and the exchange sytem will operate in the direction of Ca^{2+} efflux. At membrane potentials more positive than $E_{Na/Ca}$, the exchange current will be outwards and net Ca^{2+} influx will occur. For typical values of intra- and extracellular concentrations of Na^+ and Ca^{2+} ($[Na^+]_o$=140 mmol l^{-1}, $[Ca^{2+}]_o$=2 mmol l^{-1}, $[Na]_i$=6 mmol l^{-1} and $[Ca^{2+}]_i$=0.1 μmol l^{-1}), $E_{Na/Ca}$ is approximately −12 mV. Thus, for this example, the driving force for Na^+/Ca^{2+} antiport at a resting potential (E_m) of −70 mV will be in the direction of net Ca^{2+} efflux ($E_{Na/Ca}-E_m$=+58 mmol l^{-1}). Because of the cubic relationship in equation 1, relatively small changes in $[Na^+]_i$ can markedly affect the driving force of Ca^{2+} movements *via* the antiporter. For example, in rat cardiac muscle, $[Na^+]_i$ is substantially higher (15 mmol l^{-1}) than for other mammalian species. In this case, $E_{Na/Ca}$ is −83 mV and the antiporter should catalyze net Ca^{2+} influx in resting cells ($E_{Na/Ca}-E_m$=−13 mV), as has been observed experimentally (Shattock and Bers, 1989).

The effects of antiport activity on $[Ca^{2+}]_i$ in an intact cell are much more complicated

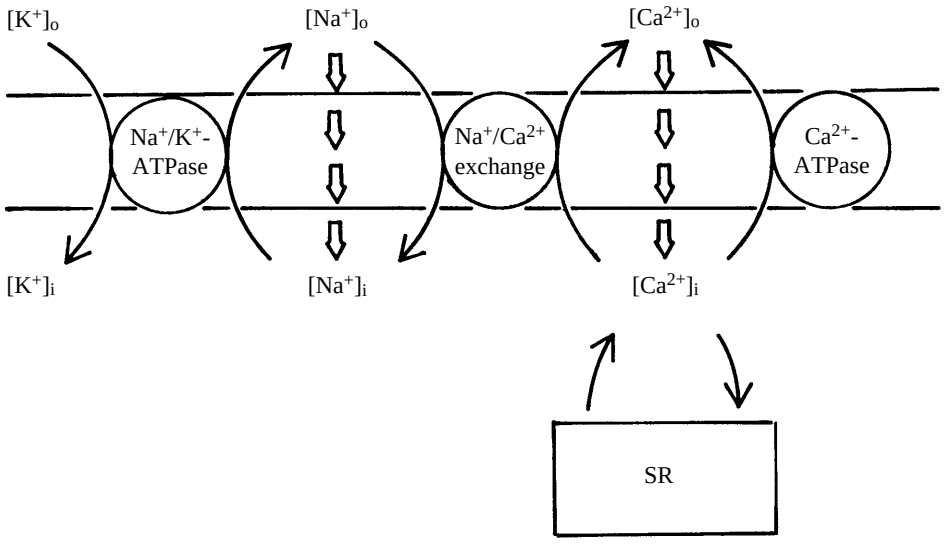


Fig. 1. Ionic homeostasis in cardiac myocytes. The transmembrane $[Na^+]$ and $[K^+]$ gradients are maintained by the operation of the Na^+/K^+-ATPase. The open arrows represent the entry of Na^+ and Ca^{2+} through voltage-gated channels. Both the Na^+/Ca^{2+} antiporter and the plasma membrane Ca^{2+}-ATPase compete for cytosolic Ca^{2+} with the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase; this competition is one determinant of the amount of Ca^{2+} stored in the SR, and has an important effect on the force of cardiac contraction. Reproduced from Reeves (1985) with permission.

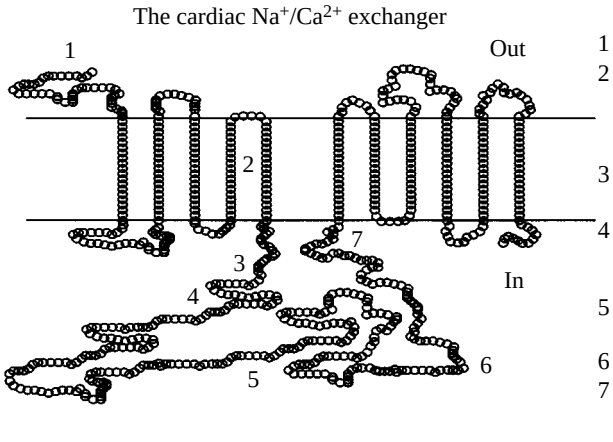
than thermodynamic considerations (equation 1) suggest. The turnover of the antiporter is regulated by the affinities of Na^+ and Ca^{2+} for transport sites as well as by secondary interactions of these ions, which regulate transitions of the antiporter between its active and inactive states. The latter regulatory processes will be discussed later in this chapter. Activation of the antiport activity by cytosolic Na^+ is highly cooperative (n=2.7) and exhibits a K_m of 18 mmol l^{-1} (Matsuoka *et al.* 1993). The K_m for Ca^{2+} at the cytosolic transport sites is approximately 4 μmol l^{-1} at $[Na^+]_o$=150 mmol l^{-1}. In cardiac cells, the resting cytoplasmic concentrations of Na^+ (4–8 mmol l^{-1}) and Ca^{2+} (40–100 nmol l^{-1}) are substantially below their respective K_m values for Na^+/Ca^{2+} antiport, suggesting that the antiporter is operating at only a small percentage of its maximal capacity. Even during contraction, $[Ca^{2+}]_i$ rarely exceeds 1 μmol l^{-1}. Transient local concentrations of Ca^{2+} immediately adjacent to the cytoplasmic sarcolemmal surface may be substantially higher than those of the bulk cytosol, however. Transient elevations of $[Na^+]_i$ appear to drive net Ca^{2+} influx *via* the antiporter during the early portions of the cardiac action potential; this transient Ca^{2+} influx is thought to contribute to triggering Ca^{2+} release from the sarcoplasmic reticulum (Leblanc and Hume, 1990). These local fluctuations in $[Na^+]_i$ have been postulated to occur within a subsarcolemmal space (dubbed 'fuzzy space'; Lederer *et al.* 1990) that equilibrates relatively slowly with the bulk cytoplasm. A possibly related observation is that antiport-mediated $^{45}Ca^{2+}$ fluxes in cardiac myocytes *in vitro* appear to involve a subcellular compartment that equilibrates rapidly with the

extracellular space (Post *et al.* 1993). Thus, the functioning of the antiporter in cellular Ca^{2+} homeostasis cannot be fully appreciated by thermodynamic and kinetic analyses alone; the influence of cellular architecture must also be considered. Unfortunately, as implied by the term 'fuzzy space', subcellular compartmentation of ion movements is only poorly understood at present.

Primary structure

The cardiac antiporter was first cloned by Philipson and his colleagues (Nicoll *et al.* 1990), who screened a λgt11 library from dog ventricle with an antibody to the purified antiporter. Subsequently, cardiac-type antiporters were cloned from bovine and rat heart, rat brain, rabbit kidney and aortic smooth muscle (references cited in Nakasaki *et al.* 1993; Kofuji *et al.* 1994). The canine cardiac antiporter is a protein of 938 amino acids containing 11 hydrophobic stretches of 20 or more amino acids that presumably represent membrane-spanning regions. Between the fifth and sixth transmembrane segments lies a 520-residue hydrophilic domain, which appears to reside on the cytoplasmic surface of the membrane. The suggested orientation of the antiporter in the membrane is depicted schematically in Fig. 2; several regions indicated in the diagram are of special interest.

The NH_2 terminus of the purified antiporter (region 1, Fig. 2) was found to begin after a 32-residue segment in the deduced cDNA sequence that had the characteristics of a cleaved signal sequence (Durkin *et al.* 1991). Subsequent studies revealed directly that the *in vitro* translated protein was cleaved at the NH_2 end in the presence of pancreatic microsomes (Hryshko *et al.* 1993). The presence of a cleaved signal sequence is highly unusual in transport proteins; we are unaware of any other porter, with the exception of the retinal rod $Na^+/Ca^{2+}+K^+$ antiporter (Reiländer *et al.* 1992), which exhibits a cleaved NH_2-terminal signal sequence. The functional significance of the signal sequence is



Regions of interest
1 External N terminus; signal sequence
2 Homology to Na^+/K^+-ATPase
194–VVEVWEGLL
61 % identical to Na^+/K^+-, SERCA-, PCMA- and H^+/K^+-ATPases
3 XIP region
219–RRLLFYKYVYKRAGKQRG
4 Homology to band 3 anion transporter
263–SHVDSFLDGALVLEVDE
60 % identical to rat band 3A
5 Ca^{2+} binding domain
446–DDDIFEEDE; 498–DDDHAGIFTFE
6 Alternative splicing region (570–645)
7 Acidic region
723–EDDDDDECGEE

Fig. 2. Orientation of the cardiac Na^+/Ca^{2+} exchanger in the plasma membrane. This diagram is based on hydropathy analysis suggesting the presence of 11 transmembrane spanning segments; the external orientation of the NH_2 terminus of the protein is based on the presence of an NH_2-terminal cleaved signal sequence.

unclear; the NH_2-terminal region of the mature protein is highly charged and perhaps the signal sequence is necessary to ensure translocation of this portion of the protein to the extracellular membrane surface.

The segment designated region 2 (amino acids 180–202) in Fig. 2 exhibits 43% identity with a corresponding region in the Na^+/K^+-ATPase (Nicoll *et al.* 1990). Moreover, the position of this segment within the protein (the transmembrane segments immediately prior to a large cytoplasmic domain) is similar in the two proteins. At the C-terminal end of this region, there is a nine amino acid sequence (194–VVEVWEGLL) that is 61% identical to the corresponding regions of the Na^+/K^+-, SERCA-, PMCA- and K^+/H^+-ATPases. Furthermore, the glutamate residue in position 199 is conserved in all of these ATPases and was found to be one of the critical residues for Ca^{2+} binding in the SR Ca^{2+}-ATPase (Clarke *et al.* 1989). This residue is also critical for the antiporter, since changing it to glutamine or aspartate abolished activity (Nicoll *et al.* 1994). In contrast, the glutamate at position 196 is not conserved among the various ATPases, and changing it to glutamine had no effect on antiport activity. These studies suggest that this region is involved in ion binding and translocation by the antiporter. Other mutations found to produce inactive antiporters (S109A, S110A, T or C, E113G or D, E199Q or D, T203 V, T810 V, S818A, S838A or N842 V; Nicoll *et al.* 1994) involve polar residues that appear to be located within or near the putative transmembrane segments of the antiporter (see below). In contrast, most of the central hydrophilic domain of the antiporter can be deleted without adverse effects on the kinetics of antiport activity (see below).

Region 3 (residues 219–238) in Fig. 2 is a 20 amino acid span containing mostly basic and hydrophobic residues and resembles the calmodulin-binding domains of a variety of Ca^{2+}-dependent proteins. A synthetic peptide corresponding to this sequence binds to calmodulin and inhibits Na^+/Ca^{2+} antiport activity (Li *et al.* 1991). This peptide has been designated XIP (for eXchange Inhibitor Peptide). It seems unlikely that this segment of the antiporter functions as an auto-inhibitory domain (by analogy to similar regions in calmodulin-dependent enzymes) since Na^+/Ca^{2+} antiport activity is unaffected by calmodulin.

Region 4 (residues 263–279; Fig. 2) exhibits 60% identity to the rat erythrocyte anion antiporter (band 3a) and a 13-residue segment within this region shows an average of 48% identity with 11 other band 3 proteins. Its role in the Na^+/Ca^{2+} antiporter is unknown. It is possible that it is involved in interactions with the cytoskeleton, since both the anion and the Na^+/Ca^{2+} antiporters interact with ankyrin (Li *et al.* 1993).

The significance of the other regions designated in Fig. 2 will be discussed in later sections of this chapter.

Other Na^+/Ca^{2+} antiporters

Na^+/Ca^{2+} antiporters have been cloned from human, rat and cow heart and exhibit striking similarity to the canine heart antiporter (>90% amino acid identity). Cardiac-type antiporters have also been cloned from brain, kidney and smooth muscle (see Nakasaki *et al.* 1993; Kofuji *et al.* 1994). They each show extensive homology with the canine antiporter and differ primarily within a limited region of the central hydrophilic domain, depicted as region 6 (residues 570–645) in Fig. 2. In this region, alternative splicing

mechanisms generate the various isoforms of the antiporter found in different tissues. Analysis of genomic clones reveals the presence of two mutually exclusive exons (A and B) coding for segments of 34 (B) or 35 (A) amino acids between positions 570 and 604/5; the amino acid sequences in these two exons are 34% identical. Following this region, there are four cassette-type exons (C, D, E and F) that may be inserted in various combinations; 32 possible isoforms can be generated in this manner (Kofuji *et al.* 1994). Thus, the cardiac isoform is A-C-D-E-F, the predominant patterns in brain are A-D or A-D-F, and in smooth muscle the pattern is principally B-D (Nakasaki *et al.* 1993). This mechanism accounts for all of the isoforms of the cardiac type antiporter thus far reported.

The reasons for the existence of tissue-specific isoforms are uncertain, especially since the kinetic properties of the antiporter are not likely to be affected by alterations in the hydrophilic domain (Matsuoka *et al.* 1993). However, as discussed below, interactions with the cytoskeleton may be imporant for both functional activity and the distribution of the antiporter on the membrane surface. The importance of membrane surface distribution for antiporter function is not yet well understood. In smooth muscle cells, the Na^+/Ca^{2+} antiporter and the Na^+/K^+-ATPase appear to be closely associated with each other and with the sarcoplasmic reticulum (Moore *et al.* 1993). In ventricular myocytes, the antiporter appears to be localized primarily to the T-tubules (Frank *et al.* 1992), although a second report disputes this claim (Kieval *et al.* 1992). Perhaps the different antiporter isoforms reflect specific requirements for interactions with the cytoskeleton and for particular membrane surface distributions in specific tissues.

Tissue-specific expression may also be regulated through the use of different promoters (Lee *et al.* 1994). RACE (rapid amplification of cDNA 5′ ends) analysis of cDNAs from brain and kidney revealed three different mRNA isoforms which differed in the 5′ untranslated region (UTR) upstream from position-34. Northern blot analysis revealed that unique 5′-UTR isoforms were expressed in heart and kidney, while a third variant was expressed in a wide variety of tissues and was particularly abundant in brain.

Cloning of the retinal rod $Na^+/Ca^{2+}+K^+$ antiporter (Reiländer *et al.* 1992) revealed surprisingly little homology with the cardiac type of antiporter, although the predicted arrangement of the polypeptide chain within the membrane was similar. Significant homology was limited to two regions: a 60-residue segment (residues 130–189 in the canine cardiac sequence) spanning the second and third putative transmembrane segments in both antiporters and a 58-residue region (818–875) which includes transmembrane segment 8. These regions showed 38% and 28% sequence identity between the two proteins respectively. Mutations of some conserved residues in these two regions of the cardiac antiporter revealed four changes (G108A, P112A, S117A and E120Q) that did not affect activity, while seven additional mutations (mentioned above) resulted in loss of activity (Nicoll *et al.* 1994). Unexpectedly, one of the latter mutations (S818A) is located in a rather hydrophilic loop between transmembrane segments 8 and 9 in the cardiac antiporter; however, this residue maps to the transmembrane segment itself in the retinal rod antiporter.

Regulation of Na^+/Ca^{2+} antiport activity

Two different, but perhaps related, regulatory processes have been identified. These

processes appear to involve two different inactive states of the carrier, the first promoted by the presence of cytosolic Na^+ and the second promoted by the absence of cytosolic Ca^{2+} (Hilgemann *et al.* 1992*a*,*b*). Simulations based on this model account remarkably well for the regulatory properties of the antiporter as determined from current measurements in myocyte sarcolemmal patches.

ATP-dependent regulation

Although Na^+/Ca^{2+} antiport is not coupled to ATP hydrolysis, its kinetic characteristics are regulated by ATP. This has been established in previous studies with squid axons, barnacle muscle, vascular smooth muscle cells and cardiac myocytes (reviewed in Reeves, 1990; Hilgemann *et al.* 1992*a*,*b*). In squid axons and barnacle muscle, ATP decreases the K_m for cytosolic Ca^{2+} and for extracellular Na^+. The effects of ATP do not appear to involve a direct interaction with the exchanger protein, since ATP does not affect antiport activity in cardiac membrane vesicles.

In sarcolemmal patches, ATP attenuates the effects of cytosolic Na^+ in promoting an inactive state of the antiporter ('Na^+-dependent inactivation') (Collins *et al.* 1992; Hilgemann *et al.* 1992*b*). An example of Na^+-dependent inactivation is shown in Fig. 3. In this experiment, antiport currents were initiated in a giant membrane patch from a guinea pig ventricular cell by the addition of 100 mmol l^{-1} Na^+ to the cytosolic membrane surface. As shown, there is a nearly instantaneous rise in outward antiport current, which subsequently declines over a period of several seconds to a fraction (30 % in Fig. 3) of its initial value. When Mg^{2+}–ATP is added following inactivation, a gradual increase in antiport current occurs. Subsequent cycles of Na^+ addition show a much smaller degree of Na^+-dependent inactivation, even after removal of the ATP. Hilgemann and his colleagues (1992*a*,*b*) have suggested that binding of Na^+ at the cytoplasmic membrane surface promotes a time-dependent entry of the antiporter into an inactive

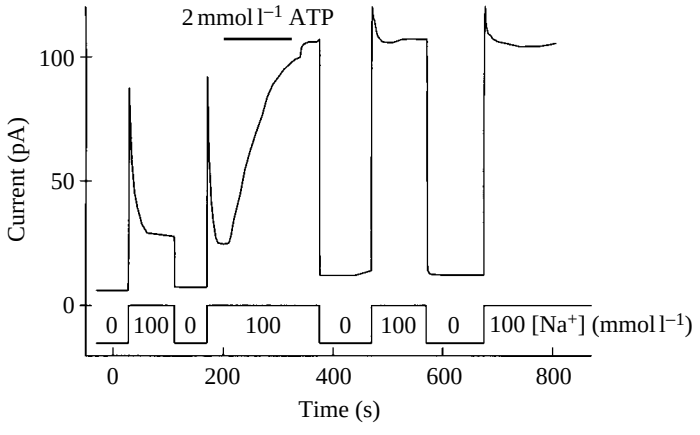


Fig. 3. Na^+-dependent inactivation of Na^+/Ca^{2+} pig ventricular sarcolemmal patches is initiated by repetitive addition of 100 mmol l^{-1} NaCl to the cytoplasmic membrane surface. As indicated by the bar, 2 mmol l^{-1} Mg^{2+}–ATP was also applied for 110 s during the second application of Na^+. Note that the decay of current during subsequent Na^+ applications was almost eliminated. Reprinted from Collins *et al.* (1992) with permission.

state. This inactivation process is reversible upon removal of Na^+ and is specific for the Na^+-bound configuration, since binding of cytosolic Ca^{2+} does not promote inactivation. Na^+-dependent inactivation is exacerbated at acidic cytosolic pH (Doering and Lederer, 1993) and can be attenuated by increasing $[Ca^{2+}]_i$.

The mechanism by which ATP counteracts Na^+-dependent inactivation is poorly understood. The evidence in squid axons suggests a phosphorylation mechanism (DiPolo and Beaugé, 1993), but antiport current measurements in cardiac sarcolemmal patches (Collins *et al.* 1992; Hilgemann and Collins, 1992) do not support this hypothesis. The latter studies suggest that the ATP effect is indirect and is mediated by an aminophospholipid translocase that maintains an elevated concentration of acidic phospholipids at the cytosolic surface of the membrane bilayer. This mechanism is consistent with the properties of the isolated antiporter, since activity is known to be stimulated by negatively charged amphiphiles, such as phosphatidylserine (Collins and Hilgemann, 1993).

ATP-dependent regulation of antiport activity has been examined in transfected Chinese hamster ovary (CHO) cells permanently expressing the bovine cardiac Na^+/Ca^{2+} antiporter (M. Condrescu, J. P. Gardner, G. Chernaya, J. F. Aceto, C. Kroupis and J. P. Reeves, in preparation). Depletion of cellular ATP using metabolic inhibitors caused a 40–50 % decline in the initial rate of antiport-mediated Ca^{2+} influx and a much more dramatic decline in extracellular-Na^+-dependent Ca^{2+} efflux. Cytosolic acidification by 0.6 pH units was observed during ATP depletion; however, restoration of the cytosolic pH in ATP-depleted cells to normal levels with NH_4Cl did not correct the decline in extracellular-Na^+-dependent Ca^{2+} efflux, although it did stimulate Ca^{2+} influx. ATP depletion also enhanced the ability of extracellular Na^+ to inhibit Ca^{2+} influx *via* the antiporter. An example of this type of experiment is shown in Fig. 4A. The K_i for $[Na^+]_o$ was reduced from 88 mmol l^{-1} to 55 mmol l^{-1} in ATP-depleted cells. The effects of ATP depletion on antiport activity were either absent or greatly reduced in CHO cells expressing a mutant form of the antiporter in which 440 out of the 520 amino acids in the central hydrophilic domain had been deleted. Thus, the effects of ATP on antiport activity appear to be mediated by the central hydrophilic domain of the antiporter.

The effects of ATP depletion were not modified or mimicked by agents that affect protein kinases or phosphatases. Furthermore, a phosphorylated form of the antiporter could not be detected when the antiporter was immunoprecipitated from ^{32}P-labelled cells, although ^{35}S-labelled antiporter was easily observed. The results imply that a phosphorylation mechanism is not involved in the effects of ATP on antiport activity.

Treatment of the CHO cells with cytochalasin D, an agent that interferes with polymerization of cytoskeletal actin, mimics the effects of ATP depletion, as shown in Fig. 4B. Cytochalasin D did not affect the extracellular $[Na^+]_o$ inhibition profile for cells expressing the deleted form of the antiporter. The results suggest that interaction of the antiporter with the cellular cytoskeleton has important functional consequences and that this may be at least partly responsible for ATP-dependent regulation of antiport activity. This interaction probably involves the central hydrophilic domain of the antiporter and might be mediated by ankyrin, a cytoskeletal protein that has been shown to bind to the cardiac antiporter (Li *et al.* 1993). This interpretation cannot explain the effects of ATP

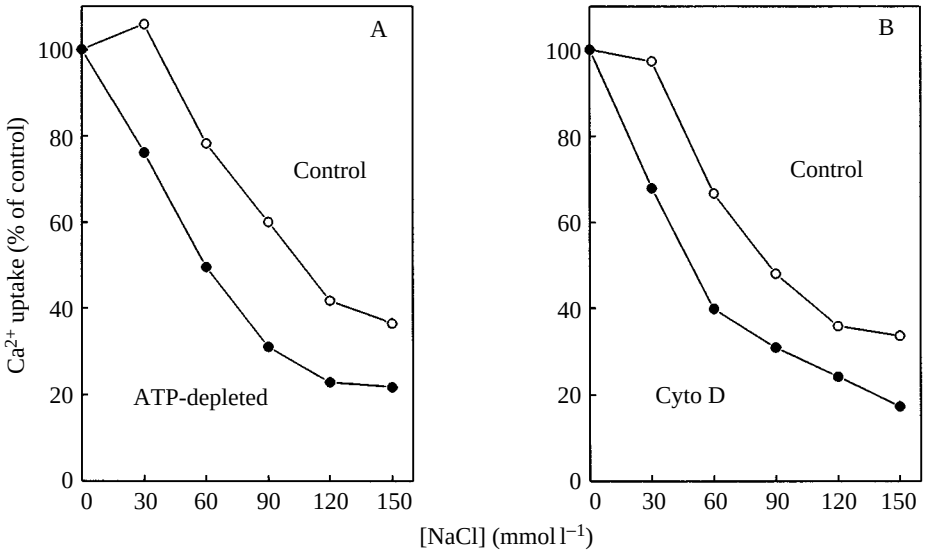


Fig. 4. Effect of ATP depletion (A) and cytochalasin D (Cyto D, B) on Na^+ inhibition of $^{45}Ca^{2+}$ uptake in transfected CHO cells. (A) CHO cells permanently expressing the bovine cardiac Na^+/Ca^{2+} antiporter were preincubated for 30 min with 0.4 mmol l^{-1} ouabain and then incubated with 2.5 μg ml^{-1} oligomycin plus 2 μmol l^{-1} rotenone for an additional 10 min; for control cells, 10 mmol l^{-1} glucose was included in the medium to maintain cellular ATP levels. The initial rate of $^{45}Ca^{2+}$ uptake was then assayed in mixtures of *N*-methyl-D-glucamine and NaCl to generate the final Na^+ concentrations shown. (B) CHO cells were preincubated for 30 min with ouabain and then assayed for the initial rate of $^{45}Ca^{2+}$ uptake as described in A. Cytochalasin-D-treated cells were preincubated with the drug (1 μmol l^{-1}) for 60 min prior to ouabain treatment and assay. The data shown are from representative experiments.

on antiport currents in giant sarcolemmal patches, however, since cytoskeletal elements appear to be absent in these patches (D. Hilgemann, personal communication).

The effects of ATP depletion and cytochalasin D were greatly reduced in a second antiporter mutant. In this mutant, a string of acidic residues in the C-terminal portion of the hydrophilic domain (723–EDDDDDECGEE; region 7, Fig. 2) were changed to alanines. This region is of interest because an even more more extensive string of acidic residues is found in the same position in the retinal rod $Na^+/Ca^{2+}+K^+$ antiporter. We initially considered the possibility that this region might serve as a means of guiding Ca^{2+} by electrostatic interactions to transport binding sites within the transmembrane domains. It was surprising, then, that antiport-mediated Ca^{2+} uptake and efflux in cells expressing this mutant did not appear to be abnormal. The alteration in regulatory properties suggests that this region may be involved, directly or indirectly, in the interaction of the antiporter with cytoskeletal elements.

Intracellular-Ca^{2+}-dependent activation

Studies with internally dialyzed squid axons, barnacle muscle and cardiac myocytes have established that an interaction of cytosolic Ca^{2+} with an activation site on the

antiporter (distinct from the Ca^{2+} transport site) is essential for 'reverse-mode' Na^+/Ca^{2+} antiport (i.e. intracellular-Na^+-dependent Ca^{2+} influx). This is also evident from current measurements in cardiac sarcolemmal patches, where the K_m for intracellular-Ca^{2+}-dependent activation is about 0.8 μmol l^{-1} (pHi=7.0; $[Na^+]_i$=18 mmol l^{-1}) and is shifted to higher concentrations by increased $[Na^+]_i$ and by removal of ATP (Hilgemann *et al.* 1992*a*). The latter observations suggest that there is an interaction between the ATP-dependent and intracellular-Ca^{2+}-dependent modes of regulation. The affinity of the intracellular Ca^{2+} activation site for Ca^{2+} increases markedly at alkaline pHi (K_{Ca}=9.6 μmol l^{-1} at pH 6.8; K_{Ca} <0.3 μmol l^{-1} at pH 7.8; Hilgemann *et al.* 1992*a*); indeed, at very high pHi (8.8), activation of the antiporter by intracellular Ca^{2+} is completely lost. Although activation by intracellular Ca^{2+} has been demonstrated repeatedly for the 'reverse mode' of antiport activity, it has not been determined whether intracellular-Ca^{2+}-dependent activation also regulates extracellular-Na^+-dependent Ca^{2+} efflux; this is difficult to determine because cytosolic Ca^{2+} is also the transport substrate in this mode of antiport activity.

Recent studies by Philipson and his colleagues (Levitsky *et al.* 1994; Hryshko *et al.* 1994) have located the sites of secondary Ca^{2+} activation to a region spanning residues 371–508 in the hydrophilic domain (region 5; Fig. 2). These investigators measured Ca^{2+} binding to portions of the hydrophilic domain expressed as fusion proteins in bacteria and to various mutants expressed in the same way. Two acidic segments (446–DDDIFEEDE and 498-DDDHAGIFTFEE) within this region were both found to be essential for high-affinity Ca^{2+} binding (K_d=0.5 μmol l^{-1}). Although the latter segment bears similarity to the Ca^{2+}-binding loop of EF hand structures, removal of the terminal E, which is essential for Ca^{2+} binding to EF hands, did not affect Ca^{2+} binding to the antiporter. Mutants that were defective in Ca^{2+} binding were also shown to be defective in secondary Ca^{2+} activation of antiport activity. Certain mutations within the two acidic segments described above led to a reduced affinity for activating Ca^{2+}. Other mutants were found in which antiport currents were independent of cytosolic Ca^{2+}. This elegant series of studies provides important new insights into the molecular mechanisms involved in the regulation of Na^+/Ca^{2+} antiport activity.

Intracellular-Ca^{2+}-dependent activation has also been observed in transfected CHO cells expressing the cardiac antiporter. As shown in Fig. 5A, intracellular loading with 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA), a Ca^{2+} chelator, inhibited Ca^{2+} influx by Na^+/Ca^{2+} antiport; these effects were not observed in cells expressing a deleted form of the antiporter missing 440 out of 520 amino acids in the hydrophilic domain (Fig. 5B). Curiously, however, when BAPTA loading was omitted and $[Ca^{2+}]_i$ was lowered (to less than 50 nmol l^{-1}) by preincubation in a Ca^{2+}-free medium, the cells exhibited an apparent *stimulation* of antiport-mediated Ca^{2+} influx compared with cells preincubated in the presence of Ca^{2+}. Similar effects were observed in cells expressing the deletion mutant. These effects did not appear to be due to an increase in $[Na^+]_i$ under Ca^{2+}-free conditions and persisted even when Ca^{2+} uptake into intracellular Ca^{2+} stores was blocked by the SERCA-ATPase inhibitor thapsigargin. The results suggest that cytosolic Ca^{2+}, in addition to activating Ca^{2+} influx at low concentrations, can also inhibit Ca^{2+} influx at higher concentrations, a phenomenon that

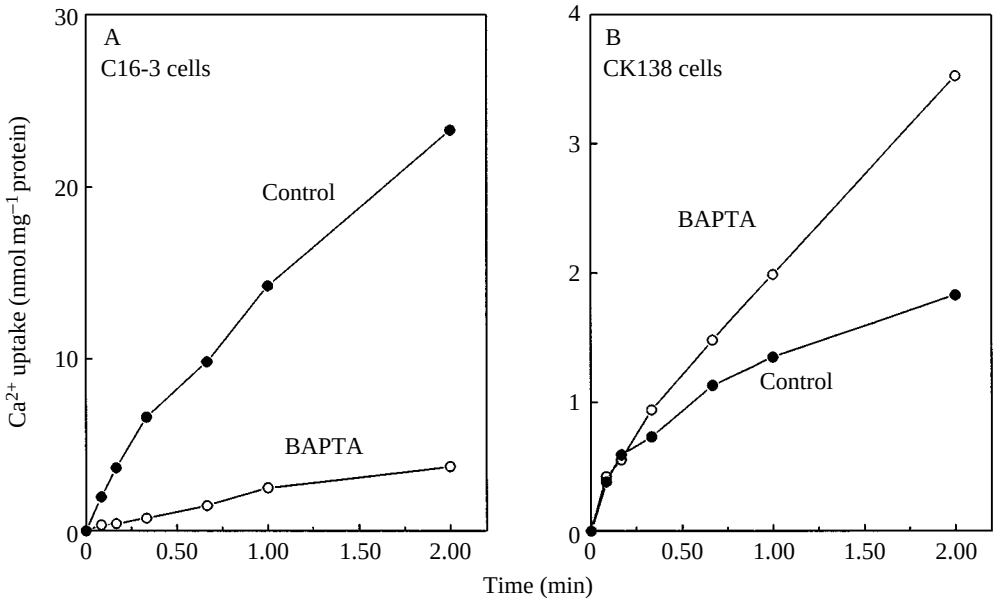


Fig. 5. Effect of intracellular BAPTA on $^{45}Ca^{2+}$ uptake by Na^+/Ca^{2+} antiport in transfected CHO cells. Cells were preincubated for 30 min with ouabain with or without 10 μmol l^{-1} BAPTA-AM and then assayed for $^{45}Ca^{2+}$ uptake in a Na^+-free medium (150 mmol l^{-1} *N*-methyl-D-glucamine). C16-3 cells (A) express high levels of the wild-type bovine cardiac Na^+/Ca^{2+} antiporter; the CK138 (B) cells express a deleted form of the antiporter missing 440 out of 520 amino residues of the hydrophilic domain. Note that total antiport activity is much lower in the CK138 cells than in the C16-3 cells. Intracellular BAPTA inhibits the initial rate of $^{45}Ca^{2+}$ uptake in the wild-type cells but not in the CK138 cells; the enhancement of uptake observed during the later portion of the time course for the CK138 cells probably reflects reduced $^{45}Ca^{2+}$ efflux due to chelation of intracellular Ca^{2+} by the BAPTA.

has also been reported for vascular smooth muscle cells (Lyu *et al.* 1992). The mechanism of the latter effect is uncertain, but it must involve a fundamentally different type of interaction from that responsible for secondary Ca^{2+} activation, since it does not require the presence of the central hydrophilic domain.

Conclusions

The major features of the Na^+/Ca^{2+} antiporter's physiological function and molecular architecture are now well understood. Two important areas for future research are discussed below.

What is the physiological significance of regulation of antiport activity?

The importance of secondary activation by Ca^{2+} is poorly understood. One possibility is that it provides a mechanism for 'turning off' Ca^{2+} efflux by the antiporter once $[Ca^{2+}]_i$ has attained a low value. In this respect, the Na^+/Ca^{2+} antiporter might be similar to the Na^+/H^+ antiporter, which is secondarily regulated by cytosolic protons. Extending the

analogy, it would be of great interest to learn whether the 'set point' for the Na^+/Ca^{2+} antiporter, like that of the Na^+/H^+ antiporter, can be altered by growth factors or hormones. This type of regulation could exert an important influence on the intracellular Ca^{2+} stores in cardiac myocytes and in other cells.

The importance of ATP-dependent regulation is even less well understood. One possibility is that the decline in antiporter function during periods of ATP depletion may protect the cells against Ca^{2+} overload. However, it seems more likely that there is an important role for this mode of regulation in normal cellular function. If ATP-dependent regulation reflects interactions with the cytoskeleton, then its importance may be tied in with locating antiport activity in proximity to particular subcellular compartments. This possibility is supported by the observation that the antiporter appears to be closely associated with the Na^+/K^+-ATPase and the sarcoplasmic reticulum in smooth muscle cells (Moore *et al.* 1993). Compartmentation of Ca^{2+} homeostatic mechanisms represents a new research frontier for which specific experimental tools and approaches are greatly needed.

Is the antiporter an important pathway for Ca^{2+} influx?

In many, if not most, cells, the thermodynamic driving force for Na^+/Ca^{2+} antiport (equation 1) favours net Ca^{2+} efflux under resting conditions. We have already discussed how this driving force might become transiently reversed during the early portions of the cardiac action potential, leading to a transient Ca^{2+} influx which can serve as a trigger for Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (Leblanc and Hume, 1990). Even when the balance of forces favours net Ca^{2+} efflux, however, there is a subtantial unidirectional Ca^{2+} influx catalyzed by the antiporter. This could constitute an important pathway for refilling or maintaining intracellular Ca^{2+} stores. In this respect, it is important to note that the total thermodynamic driving force for Na^+/Ca^{2+} antiport *plus* ATP-dependent Ca^{2+} uptake into internal stores favours the net entry of Ca^{2+} into the cell and its sequestration by internal organelles. Thus, the presence of the antiporter could promote a high level of Ca^{2+} recycling through the cell and its intracellular compartments. The possible consequences of this activity on the Ca^{2+} content of intracellular stores, cellular adaptability to environmental change and pathological conditions, such as essential hypertension, need further exploration.

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