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Na^+/Ca^{2+} Exchange and Cellular Ca^{2+} Homeostasis

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The Na^+/Ca^{2+} exchange system is the primary Ca^{2+} efflux mechanism in cardiac myocytes, and plays an important role in controlling the force of cardiac contraction. The exchanger protein contains 11 transmembrane segments plus a large hydrophilic domain between the 5th and 6th transmembrane segments; the transmembrane regions are reponsible for mediating ion translocation while the hydrophilic domain is responsible for regulation of activity. Exchange activity is regulated *in vitro* by interconversions between an active state and either of two inactive states. High concentrations of cytosolic Na^+ or the absence of cytosolic Ca^{2+} promote the formation of the inactive states; phosphatidylinositol-(4,5)*bis*phosphate (or other negatively charged phospholipids) and cytosolic Ca^{2+} counteract the inactivation process. The importance of these mechanisms in regulating exchange activity under normal physiological conditions is uncertain. Exchanger function is also dependent upon cytoskeletal interactions, and the exchanger's location with respect to intracellular Ca^{2+}-sequestering organelles. An understanding of the exchanger's function in normal cell physiology will require more detailed information on the proximity of the exchanger and other Ca^{2+}-transporting proteins, their interactions with the cytoskeleton, and local concentrations of anionic phospholipids and transported ions.

KEY WORDS: phosphatidylinositol-(4,5)*bis*phosphate; cytoskeleton; actin; chinese hamster ovary cells; cardiac myocyte.

INTRODUCTION

The force of cardiac contraction is determined in part by the amount of Ca^{2+} that is released from the sarcoplasmic reticulum (SR) during a cardiac action potential. The Ca^{2+} content of the SR is in turn determined by the interplay between Ca^{2+} influx/efflux at the sarcolemmal membrane, and Ca^{2+} accumulation/release at the SR membrane. Ca^{2+} influx at the sarcolemma occurs primarily through voltage-gated Ca^{2+} channels, whereas the principal efflux mechanism is the Na^+/Ca^{2+} exchange system. This is a carrier-mediated process which couples the movement of 3 Na^+ ions in exchange for a single Ca^{2+} ion moving in the opposite direction. With each beat of the heart, the exchanger transports 25–30% of the Ca^{2+} released from the sarcoplasmic reticulum (SR) out of the cell (Bers *et al.*, 1996). In the steady state, this Ca^{2+} efflux is balanced by an equivalent amount which enters the cell, primarily through Ca^{2+} channels. The exchanger can transport Ca^{2+} ions in either direction, depending upon the transmembrane electrical and ionic gradients; while its principal function is to mediate Ca^{2+} efflux, several investigators have suggested that it also contributes to Ca^{2+} influx during the early portion of the action potential (see below).

Na^+/Ca^{2+} exchange activity was first described as a Na^+-dependent Ca^{2+} efflux mechanism in squid giant axons (Baker *et al.*, 1969) and cardiac atrial tissue (Reuter and Seitz, 1968). It was recognized immediately that this activity was likely to mediate the inotropic effects of cardiac glycosides, which increase cytosolic Na^+ concentrations by inhibiting the Na^+, K^+-ATPase and increase the force of cardiac contrac-

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tion. Early work with squid giant axons indicated that exchange activity was stimulated by cytosolic ATP (DiPolo, 1976; Blaustein and Santiago, 1977), and that Ca^{2+}, in addition to serving as a transport substrate, also activated exchange activity in a regulatory manner, presumably by binding to sites on the exchanger that were distinct from the sites responsible for Ca^{2+} transport (DiPolo, 1979). The development of cardiac sarcolemmal vesicles to study this transport process led to a determination of the 3 Na^+: 1 Ca^{2+} stoichiometry (Reeves and Hale, 1984). This stoichiometry implies that exchange activity generates a current, and this was verified by electrophysiological measurements in cardiac myocytes (Kimura *et al.*, 1987; Hume and Uehara, 1986; Mechmann and Pott, 1986). Vesicle solubilization and reconstitution techniques led to an early estimate of the maximal turnover number of the exchanger ($> 1{,}000\ sec^{-1}$) (Cheon and Reeves, 1988) and provided the assay that resulted in the purification of the exchange protein itself (Philipson *et al.*, 1988). In 1990, the cardiac exchanger was cloned by Philipson and his colleagues (Nicoll *et al.*, 1990) and at about the same time, Hilgemann (1989) developed the "giant patch" technique for measuring exchange currents in very large excised patches. Both of these developments are central to our present understanding of the exchanger and its regulation.

MOLECULAR CHARACTERISTICS

The cardiac Na^+/Ca^{2+} exchanger (NCX1) is a protein of 938 amino acids, containing an NH_2-terminal cleaved signal sequence and 11 transmembrane segments, with a 520 amino acid, cytosolically disposed hydrophilic domain between the 5th and 6th transmembrane segments (Nicoll *et al.*, 1990) (Fig. 1). The transmembrane regions are responsible for ion translocation. Two regions spanning the extracellular halves of transmembrane segments 2–3 and 8–9 are homologous to each other, and to similarly located areas in the retinal rod exchanger; exchanger function is highly sensitive to mutations within these regions (Nicoll *et al.*, 1996a).

Deletion of the hydrophilic domain, either by proteolysis or by site-directed mutagenesis, does not markedly affect the kinetic properties of the exchanger but eliminates all aspects of regulatory control (Matsuoka *et al.*, 1993). At the beginning of the hydrophilic domain (amino acids 218–238), there is a string of 20 basic and hydrophobic amino acids which resembles a calmodulin-binding domain. Although calmodulin has no effect on exchange activity, a peptide corresponding to this region (called XIP for eXchange Inhibitory Peptide) blocks exchange activity, suggesting that this portion of the exchanger may have an autoinhibitory function (Li *et al.*, 1991). The hydrophilic domain also contains two acidic regions (446–454 and 498–509) that bind Ca^{2+} with high affinity, and these appear to constitute the regulatory sites by which cytosolic Ca^{2+} activates exchange activity (Matsuoka *et al.*, 1995; Levitsky *et al.*, 1994) (cf. below).

Further downstream lies a region (561–681) which is the site for tissue-specific alternate splicing (Kofuji *et al.*, 1994; Nakasaki *et al.*, 1993). The intron/exon arrangement of this portion of the NCX1 gene displays two mutually exclusive exons, A and B, of 35 and 34 amino acids respectively, in conjuction with four casette-type exons (C–E) of 7, 6, 5, and 24 amino acids. Exchangers expressed in heart, brain, kidney, and skeletal muscle exhibit tissue-specific splicing patterns, although no striking functional differences between the variants have yet been detected. Since the original description of NCX1, other exchanger genes have been cloned (NCX2 and NCX3) which exhibit tissue-specific differences in expression, and modest functional differences, compared to NCX1 (Nicoll *et al.*, 1996b; Li *et al.*, 1994). Exchangers which cotransport K^+ with Ca^{2+} (stoichiometry 4 Na^+/(1 Ca^{2+} + 1 K^+)) have been described in retinal rods, brain, and platelets (Schnetkamp *et al.*, 1989; Cervetto *et al.*, 1989; Kimura *et al.*, 1993); two such exchangers have been cloned (Reiländer *et al.*, 1992; Tsoi *et al.*, 1998). The present article deals solely with the cardiac isoform of NCX1 (exons A-C-D-E-F).

IN VITRO REGULATION OF EXCHANGE ACTIVITY

Kinetic data are compatible with a consecutive mechanism for Na^+/Ca^{2+} exchange with a maximal turnover of $\sim 5{,}000\ sec^{-1}$ (Hilgemann *et al.*, 1991; Hilgemann, 1996). K_m values for cytosolic Ca^{2+} and Na^+ are 4 μM and 18 mM respectively (Hilgemann *et al.*, 1991; Matsuoka *et al.*, 1993). In Hilgemann's terminology (Hilgemann *et al.*, 1992a,b), the binding sites for Na^+ and Ca^{2+} are oriented toward either the cytosol (E_1 configuration) or the extracellular medium (E_2 configuation). Conversion of one configuration to the other requires the binding and translocation of 3 Na^+ or 1 Ca^{2+} (Fig. 2).

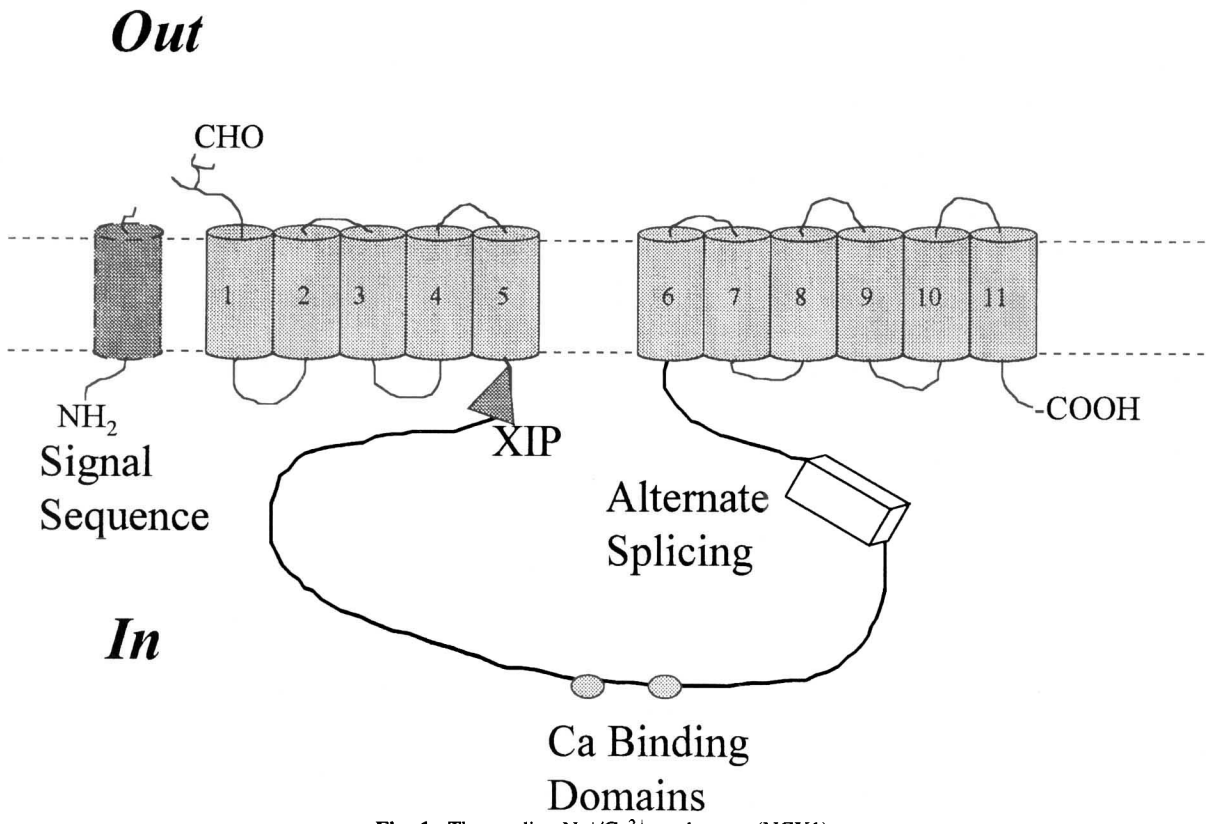

Fig. 1. The cardiac Na^+/Ca^{2+} exchanger (NCX1).

The *in vitro* regulatory behavior of the exchanger is illustrated by data in Fig. 3, which depicts current traces from "inside-out" excised giant membrane patches (Matsuoka *et al.*, 1993). When the Na^+ concentration at the cytosolic membrane surface is increased to 100 mM, there is a rapid rise in outward current

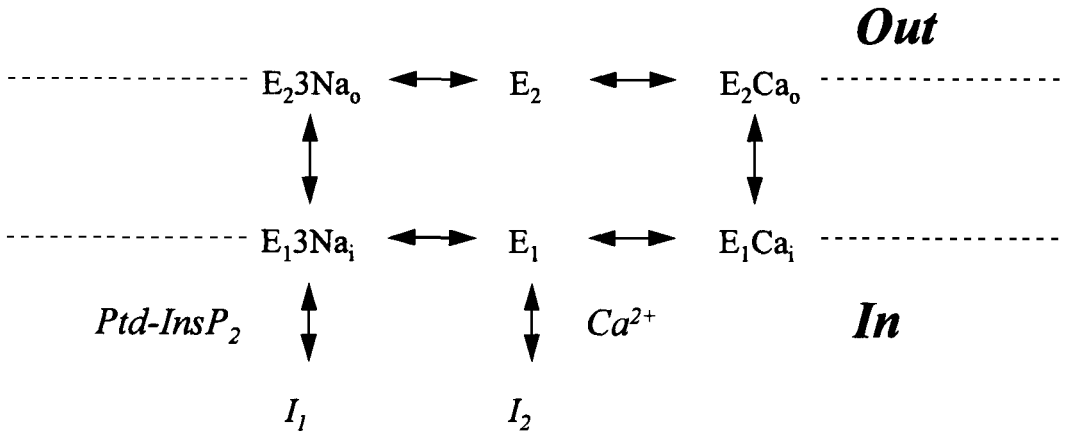

Fig. 2. Reaction mechanism of Na^+/Ca^{2+} exchange. E_1 and E_2 refer to the orientation of transport sites to the cytosol and extracellular medium respectively and I_1 and I_2 refer to the two inactive states of the exchanger (see text). I_1 inactivation is depicted as taking place from the fully Na^+-loaded configuration of the exchanger in the E_1 orientation (Hilgemann, 1990; Hilgemann *et al.*, 1992b). The exchanger configuration that gives rise to I_2 inactivation is uncertain, and is depicted here as the E_1 configuration solely for ease of representation.

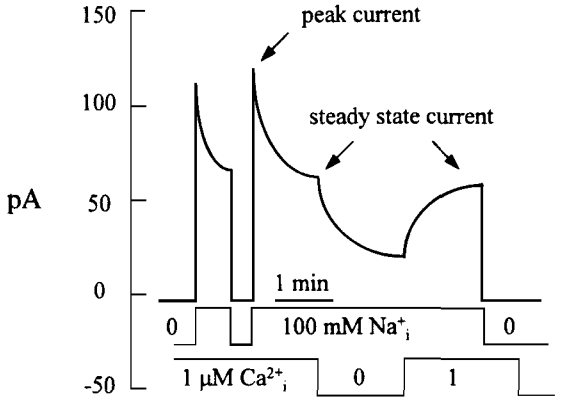

Fig. 3. Time-dependent changes in Na^+/Ca^{2+} exchange currents in excised patches illustrating I_1 and I_2 inactivation. The schematized currents are modeled from actual currents from an excised patch from *Xenopus* oocytes expressing the cardiac exchanger (Matsuoka *et al.*, 1993); reproduced with permission). An increase in cytosolic $[Na^+]$ to 100 mM produces a rapid rise in outward current to a peak value, followed by a gradual decline to a steady-state value (I_1 inactivation). In another portion of the trace, cytosolic Ca^{2+} is removed and the current declines from the steady-state value (I_2 inactivation); the current is reactivated upon restoration of cytosolic Ca^{2+}.

followed by a time-dependent decline to a steady-state value, in this instance, of approximately 60% of the peak value; steady-state currents range from 10% to nearly 100% of the peak currents, depending upon experimental conditions. In the experiment in Fig. 3, cytosolic Ca^{2+} was removed from the bath medium and then restored to the experimental concentration of 1 μM; as shown, the exchange current declined during the period of exposure to zero Ca^{2+}, and recovered upon restoration of Ca^{2+} to 1 μM; these current changes occurred with a half-time of approximately 20 s.

The regulatory mechanisms affecting exchange activity in Fig. 3 can be understood in terms of transitions from an active state to either of two inactive states (Fig. 2). Noise analysis of charge movements during exchange activity in giant patches indicates that exchangers undergo stochastic transitions between the inactive and active states, leading to small fluctuations in exchanger currents (Hilgemann, 1996). Each inactive state is thought to have a lifetime of several seconds.

The inactivation process responsible for the time-dependent decline in exchanger currents following exposure to cytosolic Na^+ (I_1 inactivation) is thought to occur when the exchanger is fully loaded with Na^+ in the E_1 configuration (Fig. 2). This process is also called "Na^+-dependent inactivation" (Hilgemann *et al.*, 1992b; Hilgemann, 1990). This mode of inactivation is counteracted by the presence of ATP and by supramicromolar concentrations of cytosolic Ca^{2+}. The effect of ATP has recently been demonstrated (Hilgemann and Ball, 1996) to be mediated by the production of phosphatidylinositol-(4,5) *bis*phosphate(Ptd-$InsP_2$), which is thought to interact directly with the exchanger. Other negatively charged amphipathic compounds, such as phosphatidylserine and even dodecylsulfate, also counteract I_1 inactivation (Hilgemann and Collins, 1992).

Experiments with squid giant axons support the notion that exchange activity might also be regulated by phosphorylation-dependent mechanisms (DiPolo and Beaugé, 1994; DiPolo and Beaugé, 1991). However, with the cardiac exchanger, protein kinase inhibitors produce either no effect (Condrescu *et al.*, 1995) or only modest stimulation (Iwamoto *et al.*, 1996; Shigekawa *et al.*, 1996) of activity in intact cells. Experiments with various protein kinases in excised patches failed to disclose significant effects of these agents on exchange currents (Hilgemann and Collins, 1992). Thus, phosphorylation does not appear to be a major mechanism of regulation for the cardiac exchanger. An exception is noted in the case of the frog heart, where cAMP-dependent mechanisms, presumably involving phosphorylation, *inhibit* exchange activity (Fan *et al.*, 1996).

The second inactive state forms in the absence of cytosolic Ca^{2+} (I_2 inactivation), and is most clearly seen as a dependence of "reverse" Na^+/Ca^{2+} exchange upon the presence of submicromolar levels of cytosolic Ca^{2+}. Note that in this mode of exchange, Na^+ is required on the cytosolic side as a transport substrate and the requirement for Ca^{2+} clearly reflects a regulatory role. Regulatory Ca^{2+} binding has been shown to activate all the modes of exchange activity: forward and reverse Na^+/Ca^{2+} exchange (Levitsky *et al.*, 1994; DiPolo, 1979; Kappl and Hartung, 1996), Na^+-Na^+ exchange (DiPolo and Beaugé, 1987; Haworth *et al.*, 1991), and Ca^{2+}-Ca^{2+} exchange (Kappl and Hartung, 1996). The requirement for regulatory Ca^{2+} can be overcome by alkaline pH, by chymotrypsin treatment (which presumably destroys the hydrophilic domain), and by site-directed mutations within the hydrophilic domain (Hilgemann, 1990; Hilgemann *et al.*, 1992a; Matsuoka *et al.*, 1993).

Cytosolic Ca^{2+} counteracts both I_2 and I_1 inactivation. In a typical current trace for Na^+/Ca^{2+} exchange in excised patches (Fig. 3), these two effects are

observed in the $[Ca^{2+}]_i$ dependences of the peak current and steady-state current respectively. Peak currents are enhanced by cytosolic Ca^{2+} over a concentration range of 300–600 nM, whereas 10-fold higher concentrations are required to counteract I_1 inactivation and elevate the steady-state current (Hilgemann *et al.*, 1992a). The high-affinity Ca^{2+} binding sites in the hydrophilic domain (see above) have been implicated in the direct activation of exchange activity, as reflected in the magnitude of the peak currents. In excised patches, activation of peak outward exchange currents by applying cytosolic Ca^{2+} takes place quite rapidly (< 0.2 s in Hilgemann *et al.*, 1992a; 0.5–1 s in Kappl and Hartung, 1996). As shown in Fig. 3, deactivation upon Ca^{2+} removal occurs more slowly (10–30 s).

An interesting variation in this response pattern has been noted for the Na^+/Ca^{2+} exchanger from *Drosophila* (Hryshko *et al.*, 1996). This exchanger, which exhibits an overall 49% identity to the mammalian NCX1, is nearly identitical to NCX1 in the two regulatory high-affinity Ca^{2+} binding regions. With the *Drosophila* exchanger, however, cytosolic Ca^{2+} *inhibits*, rather than activates, exchange activity. Presumably, the binding of Ca^{2+} to identical regulatory sites in the two types of exchangers promotes conformational changes in their hydrophilic domains that have opposite effects on substrate availability or translocation.

MODEL FOR IN VITRO REGULATION

The diagram shown in Fig. 4 depicts a speculative model which incorporates many features of the regulatory behavior of the Na^+/Ca^{2+} exchanger *in vitro*. The reader should be aware that each of the model's main features is currently under investigation and that none can be regarded as fully established. The major aspects of the model are as follows: (a) Binding of Ca^{2+} to the high-affinity "regulatory" sites on the hydrophilic domain is neccesary for ion exchange to occur. Presumably, the bound Ca^{2+} ions stabilize an exchanger configuration ("active state") that permits access of Na^+ and Ca^{2+} to their transport sites and promotes their translocation. (b) The binding of 3 Na^+ ions to transport sites at the cytosolic membrane surface promotes an intramolecular interaction of the XIP region with another part of the exchanger, presumably within the exchanger's hydrophilic domain. This region has not yet been identified, but preliminary cross-linking studies suggest that a region close to, or perhaps identical to, one of the high-affinity Ca^{2+} binding regions may be involved (Hale *et al.*, 1996, 1997). (c) In this configuration, corresponding to the I_1 inactivated state, the exchanger is unable to catalyze ion translocation. If XIP binding occurs close to the regulatory Ca^{2+} binding sites, access of Ca^{2+} to these sites may be blocked. (d) The active state is favored by the presence of Ptd-$InsP_2$, or other negatively charged phospholipids, which break the interaction between the XIP region and its intramolecular "receptor," perhaps by binding to the XIP region itself (Shannon *et al.*, 1994). Note that the interaction with negatively charged phospholipids is a natural consequence of the proximity of the XIP region to the cytosolic membrane interface. (e) High Ca^{2+} concentrations also promote the active configuration, most likely by binding to the Ca^{2+} regulatory sites and antagonizing the interaction of the XIP region with its intramolecular binding site. Because of the poor accessibility of the Ca^{2+} regulatory sites in the I_1-inactivated state, higher concentrations of Ca^{2+} are required to promote recovery from inactivation than to directly activate exchange activity. (f) Other conditions, such as high pH or proteolysis of the hydrophilic domain, destabilize the I_1 configuration of the exchanger, and promote maximal, unregulated activity.

PHYSIOLOGICAL REGULATION

What does any of this have to do with physiological mechanisms for regulating exchange activity? As discussed below, there is little direct experimental support for the involvement of either of these regulatory processes in modulating exchange activity in the normal physiological state of the cell. The I_1 inactivation process is observed experimentally at cytosolic Na^+ concentrations (100 mM) that are much higher than physiological levels; at concentrations within the physiological range (8–16 mM), Na^+-dependent inactivation is much less pronounced (Hilgemann *et al.*, 1992b). Furthermore, cellular ATP is normally maintained at levels which effectively antagonize I_1 inactivation.

Regulatory activation of exchange activity by cytosolic Ca^{2+} has a more obvious relevance to normal cardiac physiology. The slow time course (Fig. 3) for deactivation of exchange activity upon Ca^{2+} removal in excised patches suggests that the exchanger's activity would respond to diastolic and systolic $[Ca^{2+}]_i$ integrated over several beats. Thus, increases in the amount of Ca^{2+} released by the SR would stimulate exchange activity and increase Ca^{2+} efflux until SR

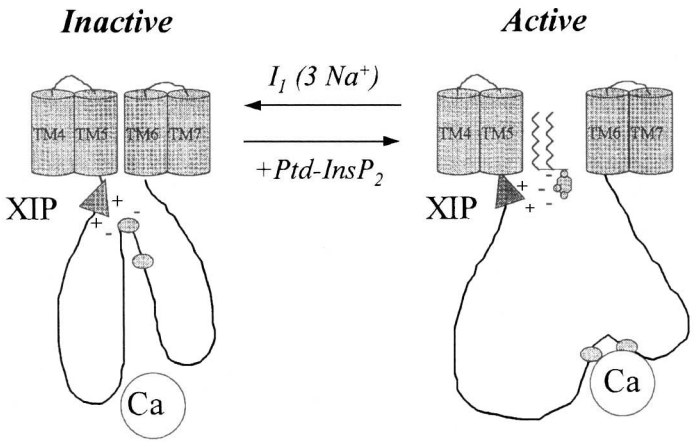

Fig. 4. Model for *in vitro* regulation of Na^+/Ca^{2+} exchange activity. Transmembrane segments 1–3 and 8–11 are not depicted in the diagram. In the inactive configuration, the XIP region is pictured as interacting with one of the high-affinity Ca^{2+} binding domains, as suggested by preliminary cross-linking studies (Hale *et al.*, 1996, 1997); the precise intramolecular binding site for XIP remains to be established, however. In the active configuration, the interaction of XIP with its intramolecular binding site is thought to be broken by the binding of acidic phospholipids to the XIP region and/or by high concentrations of Ca^{2+}. See text for further details.

Ca^{2+} stores are once again restored to an appropriately regulated level. With this picture in view, the reported concentration range for half-maximal activation of peak exchange currents in excised patches (0.3–0.6 μM) seems entirely appropriate. Modulation of the "set point" for activation of the exchanger could therefore be an important mechanism for regulatory control of cardiac contractility, and a correspondingly important focus for research.

Unfortunately, this satisfying rationale for the modulation of exchange activity by cytosolic Ca^{2+} fades away when the results obtained in more intact systems are surveyed.

Activation of Exchange Activity by Ca^{2+} in Intact Cells

Exchange activity in intact cells is activated by Ca^{2+} at concentrations 10-fold lower than in excised patches. Outward exchange currents (i.e., "reverse" Na^+/Ca^{2+} exchange) in cardiac myocytes were reported to be half-maximally activated by cytosolic Ca^{2+} concentrations of 22 and 47 nM in two separate studies (Miura and Kimura, 1989; Noda *et al.*, 1988). Measurements of exchange-mediated Ba^{2+} influx in transfected Chinese hamster ovary (CHO) cells expressing the bovine cardiac Na^+/Ca^{2+} exchanger indicated that exchange activity was accelerated by the release of Ca^{2+} from intracellular stores, and that, in agreement with the myocyte results, Ca^{2+} concentrations of 20–50 nM were sufficient to activate exchange activity (Vázquez *et al.*, 1997; Condrescu *et al.*, 1997). More extensive studies with the transfected CHO cells, as yet unpublished, demonstrate that half-maximal activation of Ba^{2+} influx by cytosolic Ca^{2+} occurs at 44 nM.

The basis for the increased Ca^{2+} sensitivity of intact cells is not understood. Local $[Ca^{2+}]_i$ gradients might elevate $[Ca^{2+}]_i$ in the vicinity of the plasma membrane substantially above that predicted from bulk cytosolic concentrations. This cannot be due solely to leakage from intracellular Ca^{2+} stores, since, in the CHO cell studies, the stores were empty. The most likely source for locally elevated $[Ca^{2+}]_i$ is the negative membrane surface charge, or the negatively charged matrix of cytoskeletal proteins underlying the plasma membrane. It is also possible that endogenous factors present in the cells, but absent from the excised patches, increase the exchanger's sensitivity to Ca^{2+}. Unfortunately, initial attempts to characterize factors that might affect Ba^{2+} influx in transfected CHO cells have been disappointing: No striking changes in $[Ca^{2+}]_i$ sensitivity were observed when variations were

introduced in cellular ATP levels or when various protein kinase inhibitors were applied.

Whatever the explanation for the exchanger's high sensitivity to Ca^{2+} in intact cells, the results raise questions as to the physiological relevance of regulatory Ca^{2+} activation. With resting $[Ca^{2+}]_i$ levels of 100 nM or so, the exchanger would be nearly fully activated and higher $[Ca^{2+}]_i$ levels (i.e., during systole) would produce little further enhancement of activity. These considerations, coupled with the previous discussion of I_1 inactivation, underscore the lack of experimental support for a well-defined mode of exchanger regulation under physiological conditions. The possibility that exchange activity is activated by interaction with Ptd-$InsP_2$ (Hilgemann and Ball, 1996) should provide an exciting new focus for addressing this issue in intact cells.

Other, less tangible aspects of exchanger function in intact cells, such as the interaction of the exchanger with cytoskeletal structures or Ca^{2+}-sequestering organelles, and the possible existence of submembrane areas of restricted ion diffusion, may prove crucial for understanding the regulation of exchange activity and its role in cellular Ca^{2+} homeostasis. We will address these issues in the remaining sections of this article.

The Actin Cytoskeleton and Na^+/Ca^{2+} Exchange

Philipson and his colleagues (Li *et al.*, 1993) demonstated that the Na^+/Ca^{2+} exchanger binds to the cytoskeletal protein ankyrin. Ankyrin serves as a bridge between certain membrane proteins and the cytoskeleton (reviewed by Bennett, 1992). Changes in Na^+/Ca^{2+} exchange activity have been linked to alterations in the actin cytoskeleton (Condrescu *et al.*, 1995). In transfected CHO cells, ATP depletion and cytochalasin D treatment both produced a dramatic breakdown in the actin cytoskeleton and altered the concentration dependence for inhibition of $^{45}Ca^{2+}$ influx by Na^+. In cells expressing an exchanger mutant in which much of the central "regulatory" domain had been deleted, the Na^+ inhibition profile was unaffected by either ATP depletion or cytochalasin D treatment. Recent results with the CHO cells, as yet unpublished, demonstrated that exchange activity was profoundly inhibited upon the breakdown of cytoskeletal filament systems following administration of a phosphatase inhibitor (calyculin). These results imply that there may be a functional link, through the cytoskeleton, between exchange activity and cellular tension, a regulatory mechanism that would be of obvious importance for cardiac muscle. At this point, however, it is uncertain whether these cytoskeletal effects are the results of directly regulating exchanger activity, fixing the exchanger's location with respect to underlying organelles, or secondarily modulating intracellular Ca^{2+} handling pathways.

Na^+/Ca^{2+} Exchange and Intracellular Ca^{2+} Stores

The use of transfected cells expressing exchange activity has provided a means of identifying functional roles for the exchanger in relation to other Ca^{2+} homeostatic mechanisms. Nontransfected (or vector-transfected) CHO cells do not have any background exchange activity, and so effects of Na^+ on Ca^{2+} mobilization that are observed in the transfected cells, but not in the vector-transfected controls, can be logically attributed to the presence of the exchanger. Thus, in transfected CHO cells, the amplitude of the $[Ca^{2+}]_i$ transient produced by release of Ca^{2+} from internal stores is reduced by the presence of extracellular Na^+ (Chernaya *et al.*, 1996). These effects are not observed in vector-transfected control cells, and undoubtedly reflect the transport of a portion of the released Ca^{2+} out of the cell by the exchanger.

In CHO cells, as in many other cell types, Ca^{2+} release from the ER activates Ca^{2+} influx through store-operated Ca^{2+} channels in the plasma membrane, a process termed "capacitative Ca^{2+} entry" (Putney, 1991). (There is no evidence that capacitative Ca^{2+} entry occurs in cardiac myocytes. For many other cell types, however, this process is crucially important for refilling intracellular Ca^{2+} stores and for activating downstream signaling events such as gene transcription.) In transfected CHO cells, extracellular Na^+ attenuates the rise in $[Ca^{2+}]_i$ produced by capacitative Ca^{2+} influx (Chernaya *et al.*, 1996). Similar effects are observed when Ca^{2+} influx is mediated by the Ca^{2+} ionophore ionomycin (unpublished observations). Again, these effects of Na^+ are not observed in vector-transfected control cells. We have speculated that the combination of Ca^{2+} influx via store-operated Ca^{2+} channels and exchange-mediated Ca^{2+} efflux might establish a circulation of Ca^{2+} at the plasma membrane, an event that could lead to an elevation of $[Ca^{2+}]_i$ at the membrane cytosolic surface (Vázquez *et al.*, 1997). Such a local gradient could contribute importantly to Ca^{2+}-dependent processes occurring there, such as

actin remodeling, cellular locomotion, and integrin-mediated signaling events.

Spaces, Fuzzy and Otherwise

Cross talk between the exchanger and Ca^{2+} sequestering organelles is undoubtedly related to their relative locations in the cell. In cardiac myocytes, inward exchange currents elicited by Ca^{2+} released from the SR follow an accelerated time course, and appear to reflect higher $[Ca^{2+}]_i$ values, than changes in bulk $[Ca^{2+}]_i$ measured with indicator dyes (Lipp *et al.*, 1990; Trafford *et al.*, 1995). Recent studies indicate that Ca^{2+} release may occur into an area of restricted diffusion, providing the exchanger with perferential accessibility to the released Ca^{2+} (Trafford *et al.*, 1995). These findings may reflect the close proximity of the exchange carriers and the Ca^{2+} release sites. In smooth muscle cells and astrocytes, the membrane distribution of exchanger protein appears to correlate with the location of the underlying endoplasmic reticulum (Moore *et al.*, 1993; Juhaszova *et al.*, 1996). In cardiac myocytes, the exchanger is present in the T-tubular membranes, where it might also interact with the underlying sarcoplasmic reticulum (Frank *et al.*, 1996; Kieval *et al.*, 1992; Sacchetto *et al.*, 1996). These apparent proximity relations are far from settled, however: Recent measurements of exchange currents and Ca^{2+} channel inactivation brought about by SR Ca^{2+} release suggest that exchange carriers may be effectively *excluded* from regions immediately adjacent to Ca^{2+} channels in cardiac myocytes (Adachi-Akahane *et al.*, 1996).

The interaction between the exchanger and the ER also operates in reverse. Depolarization of the myocyte membrane to +50 mV during the upstroke of the action potential reverses the driving force for Na^+/Ca^{2+} exchange, leading to a transient period of exchange-mediated Ca^{2+} influx. Several investigators have suggested that this Ca^{2+} could help trigger Ca^{2+}-induced Ca^{2+} release from the SR, and thus be an important component of excitation-contraction coupling. Others, however, have maintained that this mechanism is of little importance relative to the Ca^{2+} influx mediated by voltage-dependent Ca^{2+} channels. Articles by Grantham and Cannell (1996) and by Levi and Issberner (1996) provide two recent, and rather different, viewpoints of the issues involved, as well as detailed literature citations. Local heterogeneities in cytosolic Na^+ could also contribute to exchange-mediated Ca^{2+} influx during the early portions of the cardiac action potential. Activation of Na^+ channels elicited Ca^{2+} release from the SR in cardiac myocytes even though Ca^{2+} channels were blocked; the results were thought to reflect the build-up of cytosolic Na^+ and consequent activation of Ca^{2+} influx via the exchanger (Leblanc and Hume, 1990). The amount of Na^+ entering the cells in these experiments was too small to elevate bulk cytosolic $[Na^+]_i$ significantly, and it was suggested that this Na^+ must accumulate in a "fuzzy space" of restricted diffusion underlying the sarcolemma (Lederer *et al.*, 1990). Several lines of indirect evidence have been advanced in support of this concept (reviewed by Carmeliet, 1992), and recent measurements of cytosolic Na^+ by electron probe microanlysis indicate that exchange activity may generate heterogeneously distributed elevations in $[Na^+]$ (to 40 mM or more) within 20 nm of the cytosolic membrane surface (Wendt-Gallitelli *et al.*, 1993). Na^+ concentrations of this magnitude could activate "reverse" exchange activity, but might also initiate I_1 inactivation and "turn off" exchangers in their vicinity.

SUMMARY AND CONCLUSIONS

Understanding the ways in which $Na+/Ca^{2+}$ exchange activity is regulated is likely to lead to new insights into the control of cardiac contractility. The *in vitro* studies of exchanger regulation in excised patches have emphasized alterations in exchange activity arising from the transport substrates themselves: Na^+-dependent inactivation and regulatory Ca^{2+} activation. It is still unclear, however, whether these regulatory processes participate in controlling exchanger function under physiological conditions. The recent finding that exchange activity can be activated by Ptd-(4,5)$InsP_2$ should provide an exciting focal point for future studies.

We are just beginning to understand the complex functions of Na^+/Ca^{2+} exchange in intact cells. The important influence of exchange activity on the amount of releasable Ca^{2+} within intracellular stores seems clear. Local Na^+ and/or Ca^{2+} gradients and preferred pathways for Ca^{2+} movements involving intracellular Ca^{2+} stores may be important considerations when assessing the exchanger's physiological function. Another speculative consideration involves the role of the cytoskeleton, both in locating the exchanger within the membrane so that it can participate most effectively

in these pathways, and perhaps in regulating exchanger function more directly.

If these suggestions are ever to leave the realm of speculation and be subject to rigorous experimental testing, we need to develop new tools to study local processes and compartmentation in intact cells. While this article was being written, a pioneer in the development of such tools, Frederic Fay, died of a sudden heart attack while attending a scientific meeting. Dr. Fay's contributions to optical microscopy, image analysis, and the use of fluorescent probes to study Ca^{2+} signaling in smooth muscle cells were many and varied. His meeting presentations were dazzling in their technical virtuosity and conceptual novelty. Most importantly, however, he stimulated new ways of thinking about the dynamics of cellular Ca^{2+} and its role in cellular physiology. He will be greatly missed.

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