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Topical Review

Regulatory Function of Na-Ca Exchange in the Heart: Milestones and Outlook

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Milestones

The Na^+/Ca^{2+} exchange is a countertransport mechanism located in the cell membrane of almost every mammalian cell type. It can transport Ca^{2+} across the membrane and against the electrochemical gradient for Ca^{2+} by utilizing the electrochemical gradient for Na^+. Thirty years ago a Na^+/Ca^{2+} exchange mechanism was identified in the squid giant axon and in heart muscle (Baker et al., 1968; Reuter & Seitz, 1968). By measuring $^{45}Ca^{2+}$ efflux evidence for a Na^+ and Ca^{2+} countertransport system was found (Reuter & Seitz, 1968). Interestingly, an interdependence of Na^+ and Ca^{2+} had been known for some time before these crucial experiments were carried out. Several studies had reported that contractility in the heart depended on the ratio of Ca^{2+}: Na^+ in the experimental solution. Before the exchange mechanism had been discovered, these observations were interpreted to mean some form of antagonism between Na^+ and Ca^{2+}, possibly competition for a common receptor (Wilbrandt & Koller, 1948; Lüttgau & Niedergerke, 1958). Meanwhile, our knowledge about the function of the Na^+/Ca^{2+} exchange in the heart and other tissues has dramatically increased, aided by the development and application of new methods and techniques to investigate this transporter. Several excellent books and reviews cover many aspects of Na^+/Ca^{2+} exchange (Carafoli, 1985; Eisner & Lederer, 1985; Allen, Noble & Reuter, 1989; Blaustein, DiPolo & Reeves, 1991; Philipson, Nicoll & Li, 1993; Hilgemann, Philipson & Vassort, 1996; Khananshvili, 1998).

The purpose of this review is to present an introductory overview for readers entering the field of Na^+/Ca^{2+} exchange research. The emphasis is on the role of the Na^+/Ca^{2+} exchanger in cardiac Ca^{2+} signaling and its contribution to membrane current in cardiac myocytes. In addition, we point out several ongoing controversies as well as recent developments that promise to provide new approaches for carrying Na^+/Ca^{2+} exchange research into the next millennium and, ultimately, from the "molecule to the bedside".

After the identification of the Na^+/Ca^{2+} exchange mechanism research initially concentrated on the steady-state kinetics, the stoichiometry and electrogenicity of this transporter (e.g., Baker et al., 1969; Blaustein & Bantiago, 1977; Horackova & Vassort, 1979; Mullins, 1984). Important results were obtained with the voltage-clamp technique in multicellular cardiac preparations but also with sarcolemmal vesicle preparations (Philipson, Behrson & Nishimoto, 1982; Reeves & Poronnik, 1987). Today, an electrogenic stoichiometry of 3 Na^+ : 1 Ca^{2+} is generally accepted (Reeves & Hale, 1984; Eisner & Lederer, 1985). Several early studies were carried out to investigate the influence of Na^+/Ca^{2+} exchange on cardiac contraction and relaxation. Slow and tonic contractions were found to be mediated by the Na^+/Ca^{2+} exchange running in the Ca^{2+} influx mode (*for review see* Eisner & Lederer, 1985) and the importance of this transporter for Ca^{2+} removal and as a modulator for twitch tension was recognized (O'Neill, Valdeolmillos & Eisner, 1988; Bers & Bridge, 1989). With the advent of the patch-clamp technique, the current generated by the Na^+/

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Ca^{2+} exchanger (I_{NaCa}) was identified in isolated single cells and many features of I_{NaCa} were explored in the following years (Yau & Nakatani, 1984; Hume & Uehara, 1986*a,b;* Kimura, Noma & Irisawa, 1986; Mechmann & Pott, 1986). More recently, the introduction of the giant-patch technique provided several additional experimental advantages and allowed a number of studies on the Na^+/Ca^{2+} exchanger that would not have been possible without this ingenious approach (Hilgemann, 1989). For example, this technique provides free access and rapid solution control on the cytoplasmic side of the cellular membrane together with fast voltage control of a relatively large excised membrane area (10–40 μm in diameter). It came as a surprise when in 1989 it turned out that the exchanger of the retinal rod outer segment (ROS) has a stoichiometry different from the cardiac Na^+/Ca^{2+} exchanger (4 Na^+ : 1 Ca^{2+} + 1 K^+; Cervetto et al., 1989; Schnetkamp, Basu & Szerencsei, 1989; Schnetkamp, Basu & Szerencsei, 1991). Later, it turned out to be a completely different protein (Reilander et al., 1992) although some structural similarities seem to exist (Porzig & Gutknecht, 1993). K^+ cotransport was subsequently confirmed not to occur in the cardiac Na^+/Ca^{2+} exchange (Yasui & Kimura, 1990; Crespo, Grantham & Cannell, 1990). Improvements in the methods to detect and image intracellular Ca^{2+} with fluorescent indicators opened the door for studies correlating the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ with I_{NaCa}, or in other words Ca^{2+} transport with membrane current (Beuckelmann & Wier, 1989; Bers, Lederer & Berlin, 1990; Lipp & Niggli, 1994). For the analysis of experimental data, mathematical models of the Na^+/Ca^{2+} exchanger were developed and implemented both at the cellular (DiFrancesco & Noble, 1985; Luo & Rudy, 1994) and molecular level (Johnson & Kootsey, 1985; Läuger, 1987; Hilgemann, 1988).

Another milestone in Na^+/Ca^{2+} exchange research was the cloning and sequencing of the canine cardiac Na^+/Ca^{2+} exchanger in 1990 (Nicoll, Longoni & Philipson, 1990). Subsequent work has led to the discovery of several Na^+/Ca^{2+} exchanger isoforms, in different tissues and species (Kofuji et al., 1992; Aceto et al., 1992; Komuro et al., 1992; Hryshko et al., 1996).

In the remainder of this review we will concentrate on the cardiac Na^+/Ca^{2+} exchanger. Despite the significant advancements mentioned above, we are still far from a detailed understanding of the Na^+/Ca^{2+} exchanger, not only in cardiac myocytes. But also at the cellular level, the role of the Na^+/Ca^{2+} exchanger in Ca^{2+} signaling and excitation-contraction coupling (EC-coupling) is still controversial. In addition, information regarding the physiological regulation of Na^+/Ca^{2+} exchange activity in cardiac muscle is limited. At the molecular level, very little is known about the reaction steps of the transport cycle. Although the sequence of the protein has been deduced from the cDNA, the 3-dimensional structure of this transporter is still unknown and the proposed topology of the transporter in the membrane environment is not yet completely established. The functional significance of the molecular diversity with several isoforms exhibiting a distinct tissue distribution is not yet clear. Since the Na^+/Ca^{2+} exchanger is assumed to be a suitable pharmacological target in several cardiac diseases, it seems of paramount importance to fill these gaps in the future. Recent experimental developments, such as the functional expression of the Na^+/Ca^{2+} exchanger combined with site directed mutagenesis and biophysical techniques to measure Ca^{2+} transport and I_{NaCa} should greatly facilitate future research on the Na^+/Ca^{2+} exchanger.

Calcium Signaling

Resting Calcium and Relaxation of the Calcium Transient

The Na^+/Ca^{2+} exchanger plays a central role in controlling the resting $[Ca^{2+}]_i$ in heart cells. At rest $[Ca^{2+}]_i$ is around 100 nM, 10^4 times below the extracellular Ca^{2+} concentration. Both the Na^+/Ca^{2+} exchanger and the sarcolemmal Ca^{2+} ATPase are mechanisms moving intracellular Ca^{2+} across the sarcolemma (Carafoli, 1985). While the Ca^{2+} ATPase has a higher affinity for Ca^{2+}, the Na^+/Ca^{2+} exchange has a higher transport capacity and therefore serves as the principal calcium extrusion system from beat to beat (Bridge, Spitzer & Ershler, 1988; Crespo, et al., 1990). In most species it was found that the transport rate of the sarcolemmal Ca^{2+} ATPase for Ca^{2+} extrusion is about 10 times less than the Na^+/Ca^{2+} exchanger (Barry et al., 1985; Cannell, 1991). However, after prolonged resting periods occurring in vitro $[Ca^{2+}]_i$ may drop below the equilibrium of the Na^+/Ca^{2+} exchange, presumably because of Ca^{2+} removal via the sarcolemmal Ca^{2+} ATPase.

A small amount of Ca^{2+} enters the cardiac muscle cells with each heart beat via L-type Ca^{2+} current. Depending on the species and the Ca^{2+} load of the sarcoplasmic reticulum (SR) this signal is amplified 2 to 20-fold by the Ca^{2+}-induced Ca^{2+} release mechanism (CICR; Fabiato, 1985). In the steady state this amount of trigger Ca^{2+} has to be balanced by Ca^{2+} removal from the cell. In most species only the Na^+/Ca^{2+} exchange has a transport capacity sufficient for this task. This was demonstrated by comparing the charge carried by L-type Ca^{2+} current with the charge transported by I_{NaCa} reflecting subsequent Ca^{2+} removal (Bridge et al., 1988; Bridge et al., 1990). Similarly, Ca^{2+} concentration jumps generated by flash photolysis of "caged" Ca^{2+} (e.g., DM-nitrophen) can activate I_{NaCa} with amplitudes comparable to L-type Ca^{2+} currents in cardiac myocytes (Niggli & Lederer, 1993; *see also* Fig. 1 *for experimental de-*

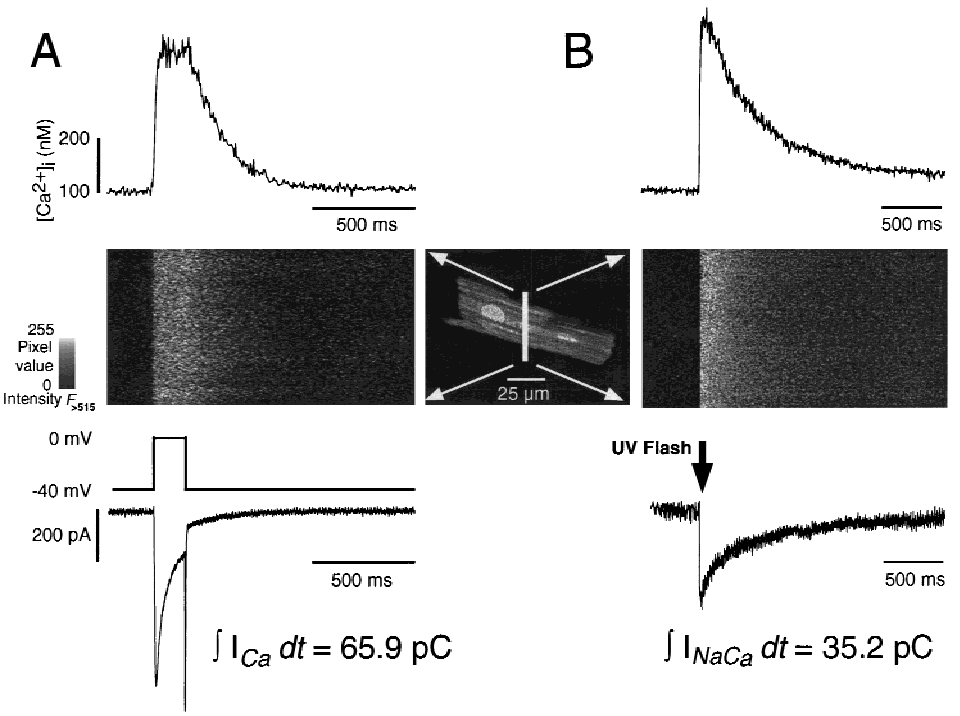

Fig. 1. Quantitative comparison of Ca^{2+} entry via L-type Ca^{2+} current (*A*) and Ca^{2+} extrusion via Na^+/Ca^{2+} exchange (*B*) in a guinea pig ventricular myocyte. To demonstrate the efficiency of the Ca^{2+} extrusion mediated by the Na^+/Ca^{2+} exchanger we induced an intracellular Ca^{2+} transient in response to a voltage activated L-type Ca^{2+} current (*A*). In the same cell transient inward I_{NaCa} was induced by an intracellular Ca^{2+} jump of similar amplitude via flash photolysis of caged Ca^{2+} (*B*). The total charge carried by I_{NaCa} and I_{Ca} was compared.

A confocal image of a fluo-3 loaded guinea pig ventricular myocyte is depicted in the center. The white line indicates the position of a single scanned line to record fluorescence *vs.* time (line-scan fluorescence image). Traces show from top to bottom: *Panel A:* Ca^{2+} transient in response to I_{Ca}, the corresponding zoomed line-scan image, voltage clamp protocol and I_{Ca}. *Panel B:* photolytic Ca^{2+} release from DM-nitrophen, the corresponding zoomed line-scan fluorescence image and I_{NaCa} at −40 mV.

If we assume that $3Na^+$ ions exchange with 1 Ca^{2+} ion and the transient Ca^{2+} entry via the L-type Ca^{2+} current is matched by transient Ca^{2+} extrusion via the transient I_{NaCa}, the predicted ratio of charge moved for an equal amount of Ca^{2+} is $\int I_{Ca}dt = 2\int I_{NaCa}dt$. A ratio of 1.8 was found which is close to the predicted value. This demonstrates that the amount of Ca^{2+} entering the cell with I_{Ca} can be rapidly extruded via the Na^+/Ca^{2+} exchanger.

I_{NaCa} and I_{Ca} were recorded in the whole cell configuration of the patch-clamp technique. For $[Ca^{2+}]_i$ measurements 0.1 µM fluo-3 (excitation: 488 nm; detection: >515 nm) was added to the pipette solution. The myocytes were pretreated with 10 µM ryanodine and 0.1 µM thapsigargin for 60 min to block the SR function. $[Ca^{2+}]_i$ was computed using a self-ratio method assuming a resting $[Ca^{2+}]_i$ of 100 nM and a K_d of 400 nM (Lipp, Lüscher & Niggli, 1996). A xenon flash lamp (230 Ws, pulse duration ≈1 msec) was used to photolyze intracellular DM-nitrophen (2 mM). Preliminary experiments were necessary to determine the precise flash intensity for a intracellular photorelease of caged Ca^{2+} with an amplitude similar to the intracellular Ca^{2+} transient in response to the voltage-activated L-type Ca^{2+} current.

tails). Consistent with the known $[Na^+]_o$- and voltage-dependence of Ca^{2+} removal by the Na^+/Ca^{2+} exchanger it was found that the rate of relaxation was steeply voltage-dependent and was significantly slowed in low $[Na^+]_o$, particularly when the SR was pharmacologically inhibited (Bridge et al., 1988; Bers & Bridge, 1989; Crespo et al., 1990).

In addition to the transporters moving Ca^{2+} across the sarcolemma into the extracellular space, there are at least two carriers that can move Ca^{2+} into intracellular organelles: (i) the SR Ca^{2+} ATPase, (ii) the Ca^{2+}-uniporter located in the mitochondrial membrane. Relaxation of the Ca^{2+} transient is thus governed by several competing Ca^{2+} transporters working in parallel. Several studies revealed that in many species the Na^+/Ca^{2+} exchanger and the SR Ca^{2+} ATPase are the most powerful transporters for Ca^{2+} removal and relaxation of the cardiac myocytes (Negretti, O'Neill & Eisner, 1993; Bassani, Bassani & Bers, 1994). There are, however, a few exceptions. For example, frog myocytes contain little SR and the Na^+/Ca^{2+} exchanger is thus very prominent (Hume and Uehara, 1986*a*,*b*), or the ferret which appears to have an exceptionally powerful sarcolemmal Ca^{2+} ATPase (Bassani et al., 1995).

Tonic Contractions

Depending on the electrochemical gradients for Na^+ and Ca^{2+} the Na^+/Ca^{2+} exchanger can also operate in the Ca^{2+} influx direction ("reverse mode"). The electrochemical gradients are governed by the intracellular and

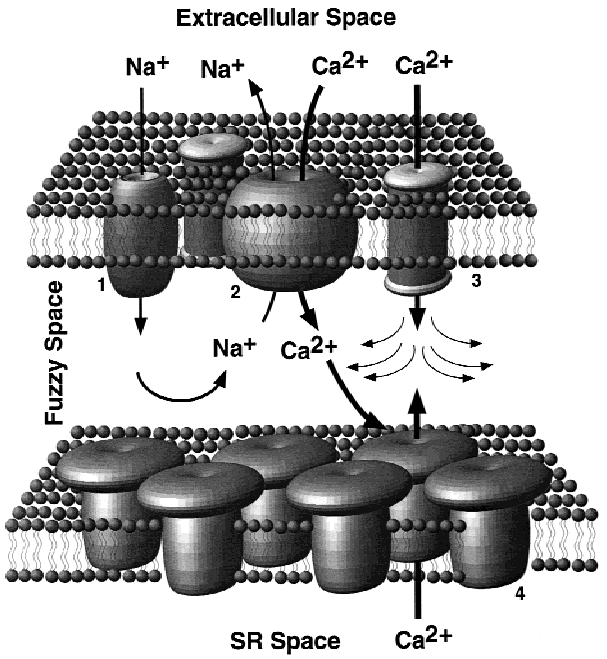

Fig. 2. Possible arrangement and close spatial association of (1) voltage-gated Na^+-channels, (2) Na^+/Ca^{2+}-exchanger, (3) L-type Ca^{2+}-channels and (4) SR-Ca^{2+}-release channels (ryanodine receptors (RyRs)) in the "fuzzy" space of cardiac myocytes (Lederer et al., 1990). In response to a transient increase of intracellular Na^+ during I_{Na}, the Na^+/Ca^{2+} exchange may generate Ca^{2+} influx into the "fuzzy" space and trigger SR Ca^{2+} release (*for details see section:* SR Ca^{2+} Release Triggered by Na^+/Ca^{2+}-Exchanger). The close spatial association of the elements in the fuzzy space may yield SR Ca^{2+} release channels with a high positive feedback since they are exposed to a high Ca^{2+} concentration during the Ca^{2+} influx. Geometries and dimensions are arbitrary.

extracellular concentrations of Na^+ and Ca^{2+}, respectively, and by the membrane potential, as described below. Thus the net flux of Ca^{2+} via the Na^+/Ca^{2+} exchanger has a reversal potential. During voltage-clamp depolarizations beyond the reversal potential the Na^+/Ca^{2+} exchanger operates in the Ca^{2+} influx mode and tonic contractions were indeed observed during such experiments (Eisner, Lederer & Vaughan-Jones, 1983). When extracellular Na^+ was reduced, the peak tonic tension increased. The application of Na^+-free solution itself elicited contractures accompanied by a membrane hyperpolarization or by an outward current. These results indicated that tonic tension in cardiac muscle is regulated by the Na^+/Ca^{2+} exchange mechanism (Horackova & Vassort, 1979). In isolated cardiac myocytes analogous tonic contractions and slow Ca^{2+} transients can be induced with strong voltage-clamp depolarizations (Bridge et al., 1988; Beuckelmann & Wier, 1989) and it is important to note that these contractions arise from a Ca^{2+} signaling pathway that is fundamentally different from the normal EC-coupling events (*see below*).

The Na^+/Ca^{2+} Exchanger and EC-Coupling

Although it is well established that cardiac muscle contraction is governed by a transient increase of $[Ca^{2+}]_i$ that is triggered by L-type Ca^{2+} current, a possible contribution of the Na^+/Ca^{2+} exchanger to this trigger signal is currently discussed (*see* Fig. 2). The main focus of this discussion is the question, whether Ca^{2+} influx mediated by the Na^+/Ca^{2+} exchanger can itself trigger Ca^{2+} release from the SR. In principle, this seems to be possible during the early phase of the action potential for two reasons: (i) during the action potential upstroke the rapid depolarization beyond the reversal potential of the Na^+/Ca^{2+} exchanger is expected to favor the Ca^{2+} influx mode and (ii) during the large Na^+ current Na^+-influx may lead to Na^+ accumulation in a restricted space under the membrane, which would accelerate any Ca^{2+} entry mediated by the Na^+/Ca^{2+} exchanger.

SR Ca^{2+} Release Triggered by Na^+/Ca^{2+} Exchange

SR Ca^{2+} release mediated by the Na^+/Ca^{2+} exchange running in the Ca^{2+} influx mode was initially reported in calcium overloaded cardiac preparations that may exhibit an increased sensitivity towards Ca^{2+} triggers (Berlin, Cannell & Lederer, 1987). Based on similar experiments it has been proposed that the Na^+/Ca^{2+} exchanger may supply a larger fraction of the trigger Ca^{2+} for CICR under physiological conditions (Levi, Brooksby & Hancox, 1993; Litwin, Li & Bridge, 1998). Additional support for this proposal came from experiments revealing sodium-current induced CICR (Lipp & Niggli, 1994).

Sodium Current (I_{Na}) Induced Ca^{2+} Release

Leblanc and Hume observed SR Ca^{2+} release after eliciting sodium currents (I_{Na}) in isolated cardiac myocytes (Leblanc & Hume, 1990). This signaling pathway had previously been overlooked because I_{Na} had usually been blocked or inactivated to avoid voltage escape during voltage-clamp experiments. The Ca^{2+} signals triggered by I_{Na} were ryanodine sensitive, required the presence of extracellular Ca^{2+} but were not inhibited by blockers of the L-type Ca^{2+} channels. It was concluded that due to a transient increase of the intracellular Na^+ concentration, the Na^+/Ca^{2+} exchange may promote Ca^{2+} influx into cardiac cells and subsequently trigger SR Ca^{2+} release. In other words, the Na^+/Ca^{2+} exchange mechanism could represent the link between Na^+ current and SR Ca^{2+} release. However, this interpretation has remained questionable and other explanations were proposed, for example spurious Ca^{2+} influx via unblocked L-type Ca^{2+} channels or via T-type Ca^{2+} channels (Sipido, Carmeliet & Pappano, 1995; Evans & Cannell, 1997) or imperfect Na^+ ion selectivity of the activated Na^+ channels

(Johnson & Lemieux, 1991; *see also* Santana, Gomez & Lederer, 1998). Taken together, this role of the Na^+/Ca^{2+} exchanger is still controversial, because some studies have failed to find evidence supporting a role of I_{Na} in triggering SR Ca^{2+} release (Sham, Cleemann & Morad, 1992; Bouchard, Clark & Giles, 1993; Sipido et al., 1995, Evans & Cannell, 1997). Differences in the density (and thus activity) of the Na^+/Ca^{2+} exchanger proteins in the sarcolemma of some species may be one explanation for the discrepancies (Sham, Hatem & Morad, 1995).

The activation of the Na^+/Ca^{2+} exchanger by the rising intracellular Na^+ concentration after I_{Na} was difficult to reconcile with straightforward assumptions. Simple calculations showed that Na^+ currents in guinea pig myocytes would elevate the bulk cytosolic Na^+ concentration only by about 25 μM, too little to significantly alter Na^+/Ca^{2+} exchange. The proposed Na^+/Ca^{2+} exchanger activation by $[Na^+]_i$ could only be explained by assuming a much smaller volume into which the Na^+ entering via I_{Na} would distribute. It was proposed that a small volume close to the membrane exists, the "fuzzy space", in which the local Na^+ concentration can become significantly more elevated during I_{Na} than in the cytoplasm (Lederer, Niggli & Hadley, 1990).

With laser-scanning confocal microscopy it was possible to detect a small Ca^{2+} signal after I_{Na} despite the presence of ryanodine. Such a signal would be expected for any of the proposed signaling pathways and presumably corresponds to the trigger signal. However, this small trigger signal had some features suggesting that it was mediated by Na^+/Ca^{2+} exchanger (e.g., it was specific for Na^+ over Li^+). The observed residual Ca^{2+} transient in the absence of SR Ca^{2+} release and L-type Ca^{2+}-current thus indicates that Na^+ current is indeed able to evoke an increase in intracellular Ca^{2+} via Na^+/Ca^{2+} exchange (Lipp & Niggli, 1994).

Electrogenicity and Stoichiometry

In early studies the transport mechanism of the Na^+/Ca^{2+} exchange was suspected to be electrogenic. Based on the cooperativity and mutual competition observed in Na^+ and Ca^{2+} flux experiments a stoichiometry closer to 3 Na^+ than 2 Na^+ for each Ca^{2+} was proposed, suggesting that the Na^+/Ca^{2+} exchanger might be electrogenic (Baker et al., 1969). Additional evidence for the electrogenicity of the Na^+/Ca^{2+} was derived from the voltage-dependence of tonic contractions measured in frog atria under voltage-clamp conditions. During these experiments tonic contractions of constant amplitude were found when the ratio of $[Ca^{2+}]/[Na^+]^3$ was kept constant (Horackova & Vassort, 1979). The observed dependence on voltage and ion concentrations again suggested a voltage-dependent and electrogenic transport mechanism for the Na^+/Ca^{2+} exchanger. Unfortunately, studies of voltage-dependence and ion competition only provide indirect information about the stoichiometry. A simple carrier model for the Na^+/Ca^{2+} exchange revealed that the voltage dependence of the Na^+/Ca^{2+} exchange does not necessarily imply electrogenicity, both electrogenic and electroneutral exchangers may be affected by membrane potential, provided the exchange cycle encompasses voltage-dependent steps (Eisner & Lederer, 1985).

Another indirect argument in favor of an electrogenic transport mechanisms was based on thermodynamic considerations. Estimates of intracellular Na^+ concentration as determined with ion sensitive microelectrodes suggested that the electrochemical Na^+ gradient was too small to account for the low intracellular Ca^{2+} concentration with less than 4 transported Na^+ ions (Mullins, 1979). Flux measurements in cardiac vesicles finally established that the exchange ratio is 3 Na^+: 1 Ca^{2+} (Reeves & Hale, 1984; *for details see below*).

Transient Inward Currents (I_{ti})

One of the first membrane currents tentatively associated with the Na^+/Ca^{2+} exchanger was the transient inward current (I_{ti}) observed in some cardiac muscle preparations. Before these currents were discovered Ca^{2+}-overloaded cardiac tissue was known to exhibit delayed after-depolarizations (DADs) and after-contractions (Ferrier & Moe, 1973). The corresponding transient inward currents were initially observed in voltage-clamped Purkinje fibers after exposure to strophantidin (Kass et al., 1978*a*). Blocking the Na^+-K^+ pump with cardiac glycosides induced a SR Ca^{2+} overload, leading to spontaneous SR Ca^{2+} release following repolarization from voltage-clamp steps. The results supported the idea that an oscillatory release of Ca^{2+} from an intracellular store was the primary event underlying both the aftercontraction and the conductance change which generates I_{ti}. The voltage-dependence of I_{ti} in Purkinje fibers exhibited a reversal potential and was outward at positive membrane potentials. Although Ca^{2+} removal via Na^+/Ca^{2+} exchanger would be consistent with a transient inward current, Na^+/Ca^{2+} exchange currents induced by a rise of intracellular Ca^{2+} should not exhibit a reversal potential (*see below*). Thus, the reversal of the current direction suggested the additional activation of Ca^{2+}-dependent ion channels (Kass et al., 1978*b;* Ehara, Matsuoka & Noma, 1989; Niggli, 1989). More recently, membrane currents observed during spontaneous SR Ca^{2+} release in cultured guinea pig atrial myocytes ("atrioballs") have also been termed I_{ti} and shown to exhibit many properties expected for I_{NaCa} (Bechem, Pott 1985, Lipp & Pott, 1988*a*). For example, the voltage-dependence of the I_{ti} did not reverse in this preparation

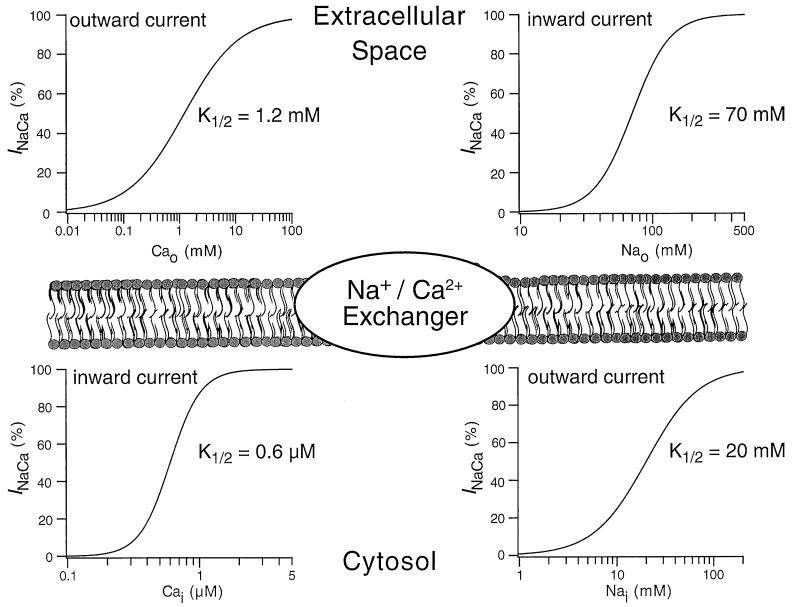

Fig. 3. Dose-response relation for activation of inward and outward Na^+/Ca^{2+} exchange current by $[Ca^{2+}]_i$, $[Na^+]_i$, $[Ca^{2+}]_o$ and $[Na^+]_I$, respectively. The inward exchange current was half maximally activated by $[Ca^{2+}]_i = 0.6$ μM, Hill coefficient $h = 3.7$ and $[Na^+]_o = 70$ mM, $h = 3.0$. The outward exchange current was half maximally activated by $[Ca^{2+}]_o = 1.2$ mM, $h = 0.87$ and $[Na^+]_i = 20$ mM, $h = 1.6$. (Kimura et al., 1987; Miura & Kimura, 1989).

and was thus compatible with the Na^+/Ca^{2+} exchanger as the charge-carrying mechanism. In addition, the organic Na^+/Ca^{2+} exchange inhibitor 3′,4′-dichlorobenzamil (DCB) blocked a membrane current component consistent with I_{NaCa} (Lipp & Pott, 1988*b*).

Creep Currents

A slowly decaying inward tail-current was sometimes seen upon repolarization in cardiac Purkinje fibers after rundown of the I_{ti} (and thus probably rundown of SR Ca^{2+} uptake and release) (Eisner & Lederer, 1979). This current also exhibited many of the properties expected for an I_{NaCa} and was termed "creep current." Similar currents were later analyzed in detail in frog atrial cells which only contain a rudimentarily developed SR (Hume & Uehara, 1986*a*,*b*). In these cells, intracellular Na^+ loading by means of an ionophore induced a slowly decaying outward current during depolarizing voltage-clamp pulses, followed by an inward current with a similar time course after repolarization. The development of the "creep current" was accompanied by cell shortening. On repolarization both the inward current and the cell length recovered in parallel, indicating that the creep current was activated by $[Ca^{2+}]_i$. In addition to elevated intracellular $[Na^+]$ the presence of extracellular Ca^{2+} was required for the development of outward creep current and the involvement of the electrogenic Na^+/Ca^{2+} exchanger was suggested as the most likely mechanism underlying both the outward and inward creep currents.

Pharmacological Isolation of I_{NaCa}

Several experimental techniques established during the last decade greatly facilitated the experimental characterization of the Na^+/Ca^{2+} exchange current. The whole-cell voltage-clamp technique in combination with intracellular dialysis was employed in the initial isolation of Na^+/Ca^{2+} exchange currents in single cardiac myocytes (Kimura et al., 1986). Such voltage-clamp studies of Na^+/Ca^{2+} exchange currents were complicated by two difficulties, particularly in cardiac myocytes: (i) during voltage-clamp depolarizations several membrane currents overlapping with I_{NaCa} are activated, some of which are also $[Ca^{2+}]_i$-dependent; (ii) no specific pharmacological blocker for I_{NaCa} is available, making it difficult to identify I_{NaCa} experimentally. Using intracellular perfusion combined with the whole-cell voltage-clamp to control and change both $[Na^+]_i$ and $[Ca^{2+}]_i$, it was possible to manipulate the substrates for the Na^+/Ca^{2+} exchange on both sides of the cell membrane. Pharmacological tools were used to block most interfering currents via known membrane channels (i.e., Ca^{2+} channels, K^+ channels). Using this approach, membrane currents were identified that showed the expected dependence on the concentration of intra- and extracellular Na^+ and Ca^{2+}, respectively. Taken together the results of several studies using the same experimental strategy revealed that inward exchange current was half maximally activated by 0.6 μM $[Ca^{2+}]_i$ or 70 mM $[Na^+]_o$, while outward I_{NaCa} required 1.2 mM $[Ca^{2+}]_o$ or 20 mM $[Na^+]_i$ for half maximal activation (*see* Fig. 3; Kimura, Miyamae & Noma, 1987; Miura & Kimura, 1989). In addition to its role as a substrate for the Na^+/Ca^{2+} exchanger, a stimulatory effect of $[Ca^{2+}]_i$ on I_{NaCa} was observed. This effect was similar to the catalytic effect of $[Ca^{2+}]_i$ described in squid axon (DiPolo, 1979), barnacle muscle (Rasgado-Flores, Santiago & Blaustein, 1989) and sarcolemmal vesicles (Reeves & Poronnik, 1987) but had a very high Ca^{2+} affinity with an apparent K_d of 22 nM

(Miura & Kimura, 1989). The affinity of this site may actually have been somewhat less because it is difficult to reach such a low $[Ca^{2+}]_i$ in intact cells via patch-clamp dialysis (Mathias, Cohen & Oliva, 1990).

Interestingly, the observed Na^+/Ca^{2+} exchange currents could be blocked by several trivalent and divalent cations like La^{3+} and Ni^{2+}. Used under the given conditions (i.e., with most other currents already blocked by more specific inhibitors), Ni^{2+} turned out to be a fairly selective tool to suppress I_{NaCa}. The general experimental strategy to block all other Ni^{2+} sensitive membrane currents and then add Ni^{2+} as a tool to suppress I_{NaCa} has subsequently found widespread application in several laboratories and in numerous voltage-clamp studies on the cellular and molecular function of I_{NaCa} (for example: Beuckelmann & Wier, 1989, Niggli & Lederer, 1991*b*).

An alternative strategy to probe the Na^+/Ca^{2+} exchange current by manipulating its substrates involves the rapid change of the intracellular $[Ca^{2+}]$ with photolabile "caged" calcium compounds (e.g., DM-nitrophen; Kaplan & Ellis-Davies, 1988). In cardiac myocytes concentration jumps of $[Ca^{2+}]_i$ induced with flash photolysis of caged Ca^{2+} elicited rapidly activating inward Na^+/Ca^{2+} exchange currents which subsequently decayed slowly, similar to inward creep currents (*see* Fig. 1 for an example). The initial peak of the inward current and the time-course of current decay were voltage-dependent and no reversal of the current direction was found, as expected for changes of I_{NaCa} induced by intracellular Ca^{2+} (Niggli & Lederer, 1993). This technique provides the unique experimental possibility to activate I_{NaCa} independently of Ca^{2+} influx and independently of the membrane potential. In addition, the very rapid concentration jump of $[Ca^{2+}]_i$ may allow presteady state studies of the Na^+/Ca^{2+} exchanger molecules (Niggli & Lederer, 1991*a;* Kappl & Hartung, 1996). The giant-patch technique is another recent development with a significant impact for Na^+/Ca^{2+} exchanger research. This technique allows recording of currents from a large membrane patch sealed to a large-pore patch pipette (Hilgemann, 1989). This arrangement has a very low electrical series resistance allowing current recordings at a high frequency bandwidth (Lu et al., 1995) combined with rapid access of experimental solutions to the cytoplasmic side of the exchanger. The surface area of the giant patch is sufficiently large to record currents not only from membrane channels but also from electrogenic transporters, such as the Na^+/Ca^{2+} exchanger or the Na^+-K^+ ATPase. Since its introduction, the method has allowed a detailed characterization of many aspects of Na^+/Ca^{2+} exchanger function, particularly when applied in combination with mutagenesis studies (*see below*).

A Mathematical Description of I_{NaCa}

For a qualitative description of the Na^+ and Ca^{2+} sensitivity of the Na^+/Ca^{2+} exchange current and the resulting current-voltage relations the model suggested by DiFrancesco and Noble has been used successfully in several studies (DiFrancesco & Noble, 1985). This formulation is useful to analyze some functional characteristics of the Na^+/Ca^{2+} exchanger fluxes and currents but in this model the regulatory and catalytic effects of the transported ions (e.g., intracellular Ca^{2+}) were not taken into account. The Na^+/Ca^{2+} exchange current is approximated by the following equation:

$$I_{NaCa} = k\{([Na^+]_i^3[Ca^{2+}]_o \exp(rE_mF/RT) - [Na^+]_o^3[Ca^{2+}]_i \exp(-(1-r)E_mF/RT)\}$$

where k is a scaling factor, E_m is the membrane potential, i and o denote the intra- and extracellular ion concentrations, respectively. The partition coefficient r represents the position of a single energy barrier located in the electrical field across the cell membrane and determines the steepness and symmetry of the voltage dependence. The overall I_{NaCa} is the difference of the unidirectional outward current component (first term, dependent on $[Na^+]_i$ and $[Ca^{2+}]_o$) and the unidirectional inward current component (second term, dependent on $[Na^+]_o$ and $[Ca^{2+}]_i$). Outward and inward current (i.e., flux) are always generated simultaneously, unless the concentration of one of the transported substrates is zero. If the unidirectional outward current component is larger than the inward current component the net measured current will be outward, corresponding to net Ca^{2+} influx via the Na^+/Ca^{2+} exchanger. Conversely, a net inward current is found under conditions where the exchanger is working in the Ca^{2+} efflux mode. This equation also predicts the reversal potential (E_{NaCa}) for the net I_{NaCa}. Assuming a 3 Na^+: 1 Ca^{2+} stoichiometry, the reversal potential is

$$E_{NaCa} = 3\,E_{Na} - 2\,E_{Ca}.$$

We have used these equations to give a short overview how the extra- and intracellular substrate concentrations will affect the reversal potential, voltage dependence and amplitude of unidirectional fluxes as well as the Na^+/Ca^{2+} exchange currents (Fig. 4).

Intracellular Ca^{2+}

During cellular Ca^{2+} signaling events and changes of the membrane potential the activity of the Na^+/Ca^{2+} exchanger will be profoundly affected. Increasing $[Ca^{2+}]_i$ accelerates Ca^{2+} efflux (shown as an unidirectional (inward) current component in Fig. 4*A*). Note the voltage-dependence of the inward current and the important feature that any difference current induced by elevations of $[Ca^{2+}]_i$ will always be inwardly directed and never reverse. This corresponds to a typical experimental situation under whole-cell voltage-clamp when the exchange current is changed by a spontaneous SR Ca^{2+} release (Berlin, Cannell & Lederer, 1989) or by a $[Ca^{2+}]_i$ jump

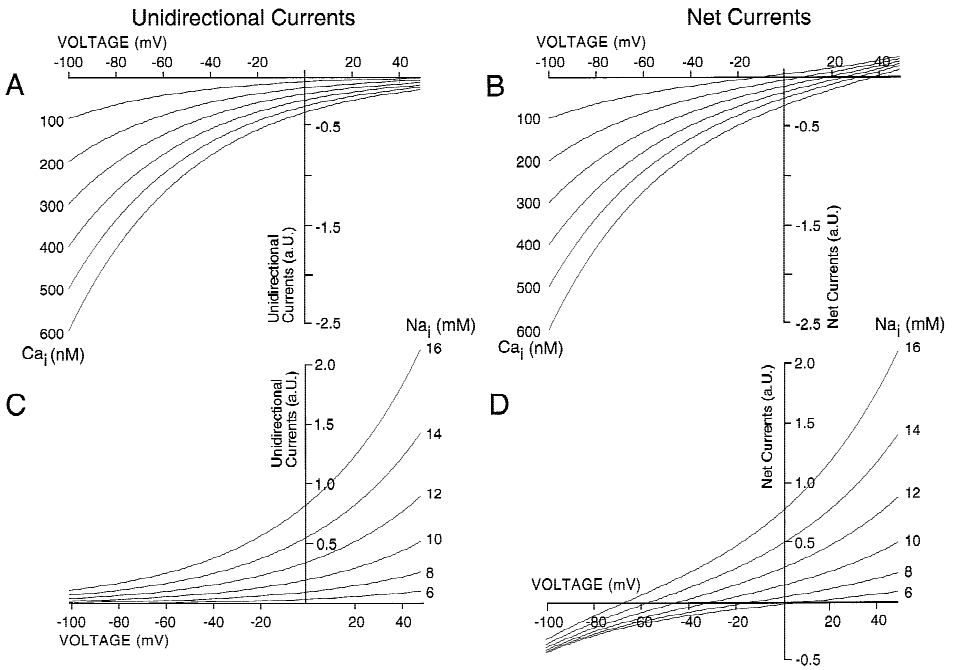

Fig. 4. Model calculations of the Na^+/Ca^{2+} exchange current. Calculations are based on the DiFrancesco and Noble model (*see* A Mathematical Description of I_{NaCa} for details). $F/RT = 38.96\ V^{-1}$; partition coefficient = 0.5 (symmetry of the reversal potential); scaling factor = 0.2 A $mol^{-4}l^4$ (generates currents close to experimental data); stoichiometry: $n = 3$; the potential is running from −100 to +50 mV. (*A*) An increase of $[Ca^{2+}]_i$ results in larger unidirectional inward currents corresponding to Ca^{2+} efflux. The net currents also show a shift of the reversal potential to more positive voltages. (*B* and *C*) An elevation of $[Na^+]_i$ favors influx of Ca^{2+}, shown as an unidirectional outward current. The reversal potential is shifted to more negative voltages (*D*). (*A* and *B*) $[Ca^{2+}]_i$ = 100–600 nM, $[Ca^{2+}]_o$ = 2 mM, $[Na^+]_o$ = 140 mM, $[Na^+]_i$ = 10 mM. *C* and *D:* $[Na^+]_i$ = 6–16 mM, $[Na^+]_o$ = 140 mM, $[Ca^{2+}]_i$ = 100 nM; $[Ca^{2+}]_o$ = 2 mM.

induced with flash photolysis of "caged" Ca^{2+} (Niggli & Lederer, 1991; *see also* Fig. 1*B*). The voltage-dependence of the Ca^{2+}-induced unidirectional I_{NaCa} corresponds to a monoexponential function with a larger inward current at more negative potentials. In contrast to the Ca^{2+}-induced change in I_{NaCa}, the **net** I_{NaCa} current (shown in panel 4*B*) exhibits an obvious reversal potential that is progressively shifted to more positive potentials at higher $[Ca^{2+}]_i$. At potentials positive to the reversal, net Ca^{2+} transport would be inward.

Intracellular Na^+

There are several conditions in which $[Na^+]_i$ may change significantly, either in the entire cytosol or in the subsarcolemmal fuzzy space. For example, after inhibition of the Na^+-K^+ ATPase with cardiac steroids or during I_{Na} (*see above*). An increase of the intracellular Na^+ concentration enhances the outwardly directed Na^+/Ca^{2+} exchange current component corresponding to Ca^{2+} influx (Fig. 4*C*). In addition, the I_{NaCa} reversal potential is shifted towards more negative voltages as $[Na^+]_i$ increases (Fig. 4*D*). Taken together, elevations of $[Na^+]_i$ favor Ca^{2+} entry via the Na^+/Ca^{2+} exchanger and reduce Ca^{2+} removal. Both effects lead to an increased Ca^{2+} load of the cell and the SR resulting in positive inotropy and ultimately spontaneous SR Ca^{2+} release and arrhythmias (*see above*).

Changes of $[Ca^{2+}]_o$ or $[Na^+]_o$ would have complementary effects on the respective ion fluxes via the Na^+/Ca^{2+} exchanger. Although not usually occurring under physiological conditions, such interventions can be used experimentally to inhibit or stimulate the Na^+/Ca^{2+} exchanger.

Exchange Current Density

The functional role of the Na^+/Ca^{2+} exchanger during cardiac Ca^{2+} signaling may differ from one species to another as well as during development. For example, some species and developmental stages are known to lack a fully functional sarcoplasmic reticulum in their cardiac myocytes. Such cells would need to rely on a more efficient transsarcolemmal Ca^{2+} signaling system and thus may exhibit a larger Na^+/Ca^{2+} exchange current per surface area. This increased current density could, in principle, arise from a higher degree of protein expression or from a faster turnover rate of the Na^+/Ca^{2+} exchanger. Using whole-cell patch clamp measurements in

guinea pig ventricular myocytes a Ca^{2+}-activated Na^+/Ca^{2+} exchange current density of about 0.73–1.48 $pApF^{-1}$ was estimated (Kimura et al., 1987). But in intact cardiac muscle cells the experimental increase of $[Ca^{2+}]_i$ has to remain limited because of the mechanical force produced by the contractile proteins. Based on giant patch measurements at saturating $[Ca^{2+}]_i$ currents of 20–30 $pApF^{-1}$ were recorded for I_{NaCa} and a density of 400 μm^{-2} exchanger molecules has been estimated (Hilgemann, Nicoll & Philipson, 1991). Assuming a surface of $25 * 10^3$ μm^2 for ventricular myocytes a maximum turnover rate of 5000 sec^{-1} was derived. Flash photolysis experiments at non-saturating $[Ca^{2+}]_i$ provided a lower limit of 10 $pApF^{-1}$ for the exchange current density in guinea pig ventricular myocytes (Niggli & Lederer, 1991*b*). When directly comparing various species with the same technique significant differences were found (Sham et al., 1995). Relatively high exchange current densities were activated by caffeine-induced Ca^{2+} release in hamster, guinea pig and mouse cardiac myocytes (4.06 $pApF^{-1}$, 1.84 $pApF^{-1}$ and 1.61 $pApF^{-1}$, respectively) while human and rat myocytes had lower densities (0.93 $pApF^{-1}$ and 0.81 $pApF^{-1}$, respectively; Sham et al., 1995; Morad & Suzuki, 1997). Recently, transgenic mice overexpressing the canine cardiac Na^+/Ca^{2+} exchanger were developed (Adachi-Akahane et al., 1997). Compared to wild-type myocytes (1.61 $pApF^{-1}$) transgenic cells exhibited a three fold larger I_{NaCa} (4.96 $pApF^{-1}$) but all other cell parameters such as cell capacitance, I_{Ca} and CICR remained unchanged (Morad & Suzuki, 1997).

I_{NaCa} and the Action Potential

The Na^+/Ca^{2+} exchanger is under the influence of the membrane potential via the electrochemical driving forces for Na^+ and Ca^{2+}, but because of its electrogenicity the Na^+/Ca^{2+} exchanger itself also contributes to the action potential (Weidmann, 1993; Benardeau, et al., 1996, Janvier & Boyett, 1996; Janvier, Harrison & Boyett, 1997). Depending on species and conditions, the ventricular action potential exhibits a plateau phase with a slow repolarization lasting for about 200 msec. During the upstroke of the action potential the membrane becomes transiently more positive than the Na^+/Ca^{2+} exchange reversal potential and a brief outward Na^+/Ca^{2+} exchange current may be present until $[Ca^{2+}]_i$ has risen sufficiently. The rise in intracellular Ca^{2+} shifts the reversal potential of the Na^+/Ca^{2+} exchanger in the positive direction and thus the membrane potential becomes again more negative than the reversal. Consequently, an inward I_{NaCa} will be present as long as $[Ca^{2+}]_i$ remains elevated, i.e., during most of the plateau and repolarization.

Presumably, the Na^+/Ca^{2+} exchange current is relatively small during the depolarized plateau phase of the cardiac action potential because of its voltage-dependence. It is assumed that the inward Na^+/Ca^{2+} exchange current is in the range of 150–450 pA per cell during the ventricular action potential. But the repolarization may stimulate the inward I_{NaCa} again during the final phase of repolarization causing a second current peak (Egan et al., 1989; Noble et al., 1991). Model calculations suggest about 300 pA for the second peak of the Na^+/Ca^{2+} exchanger. Finally, the $[Ca^{2+}]_i$ will decline to resting levels and inward I_{NaCa} will become very small. (Egan et al., 1989; Noble et al., 1991; Janvier & Boyett, 1996).

As already mentioned, during the first few milliseconds of the action potential I_{NaCa} is presumably outward because the membrane potential is more positive than the reversal of the Na^+/Ca^{2+} exchange. This membrane current could even be larger because $[Na^+]_i$ may be significantly elevated during I_{Na} in the "fuzzy space." Thus the driving force for Ca^{2+} entry via the Na^+/Ca^{2+} exchange will increase due to the synergic effects of membrane depolarization and increased local Na^+ concentration. Not surprisingly, this current would be difficult to measure as it temporally overlaps with the large I_{Na}. The effect of Na^+/Ca^{2+} exchange currents on the action potential has been difficult to investigate directly, most likely because of the lack of a suitable blocker. Much of the information available today has been obtained by extrapolation from measurements of I_{NaCa} under voltage-clamp conditions and by using mathematical models of the cardiac action potential (DiFrancesco & Noble, 1985; Luo & Rudy 1994).

Stoichiometry

Even though the voltage dependence and electrogenicity of the Na^+/Ca^{2+} exchange were predicted soon after its discovery, the exact stoichiometry of the exchange mechanism was only determined several years later. Early models assumed an electroneutral 2 Na^+: 1 Ca^{2+} stoichiometry based on the observation that contractions and Ca^{2+} flux were proportional to $[Ca^{2+}]_o/[Na^+]_o{}^2$ (Lüttgau & Niedergerke, 1958) and it was suggested that two Na^+ ions competed with a single Ca^{2+} ion for a common binding site on the exchanger (Reuter & Seitz, 1968). Flux measurements in squid axons supported the idea that more than two Na^+ ions might be transported for each Ca^{2+} ion (Baker et al., 1969).

The exchange stoichiometry was determined more directly by Reeves and Hale using $^{45}Ca^{2+}$ flux measurements in a bovine heart vesicle preparation (Reeves & Hale, 1984). The measurements based on the central idea that equilibrium conditions of the exchange process could be achieved by adjustments of the Na^+ and Ca^{2+} concentrations on both sides of the membrane. Under equilibrium conditions the overall driving force is zero

and can be described by the membrane potential and the equilibrium potentials for Na^+ and Ca^{2+}. The vesicles were treated with valinomycin in K^+ containing solutions to impose positive or negative membrane potentials. The stoichiometry *n* could be determined from the magnitude of the Na^+ gradient that exactly compensated for the membrane potential. The value of *n* determined with this approach was 2.97, very close to the presently accepted value of $n = 3$.

It is possible to predict the reversal potential for the Na^+/Ca^{2+} exchange current for each stoichiometric coefficient, as outlined above. Conversely, measurements of the reversal potential of I_{NaCa} under known ionic conditions can be utilized to determine the stoichiometry of the exchanger. Using the whole-cell voltage-clamp in combination with intracellular perfusion the reversal potential of the Na^+/Ca^{2+} exchange current was measured and a 3 Na^+: 1 Ca^{2+} stoichiometry was found (Ehara et al., 1989; Beuckelmann & Wier, 1989; Crespo et al., 1990). These studies showed that changes of the Na^+ and Ca^{2+} concentration shifted the E_{NaCa} as expected for a 3 Na^+: 1 Ca^{2+} stoichiometry.

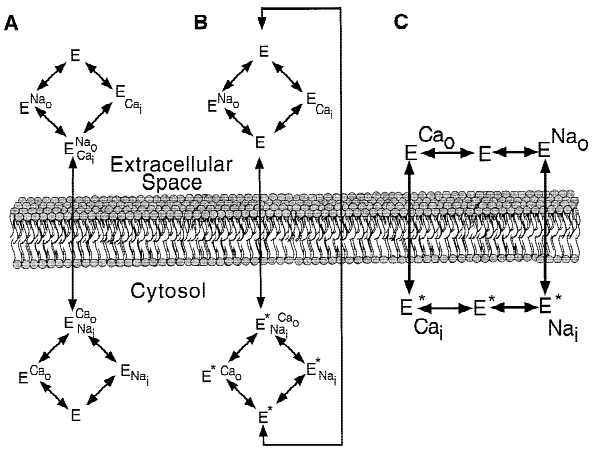

Fig. 5. Diagrams of simplified Na^+/Ca^{2+} exchange models. (*A*) simultaneous one step model. (*B*) simultaneous two step model. (*C*) cosecutive two step model. (*see also* Kinetic Models, modified from Niggli & Lederer, 1991*b*). The exchanger may either transport the substrate ions in two consecutive membrane-crossing molecular transitions (*C*) or may transport the ions simultaneously during one transition (*A* and *B*). A two-step simultaneous exchanger (*B*) has to rearrange itself (E <—> E* transition) before it can start the next transport cycle (two membrane crossing transitions, only one moves ions). A one-step simultaneous exchanger (*A*) that does not require such a molecular rearrangement before it enters the next transport cycle (one membrane crossing transition) since the exchanger has an identical configuration on both sides of the membrane.

Molecular Function and Structure

Kinetic Models

Although several cellular properties of the Na^+/Ca^{2+} exchanger have been investigated in some detail, much less is known about its function at the molecular level. In principle, two fundamentally different schemes are conceivable for the transport cycle, either a simultaneous or a consecutive transport mechanism (*see* Fig. 5). In the first case, the transporter moves the ions simultaneously during a membrane-crossing molecular transition (Fig. 5*A* and *B*). If a simultaneous exchanger has to rearrange itself before it can undergo the next transport cycle (two consecutive membrane-crossing transitions, only one transition moves ions) it is called two-step simultaneous transporter (*B*), whereas a one-step simultaneous transporter does not require an additional molecular rearrangement before it can enter the next transport cycle (*A*). A consecutive transporter moves Na^+ and Ca^{2+} in two separate membrane crossing steps (*C*).

Several groups investigated self-exchange (i.e., Na^+-Na^+ and Ca^{2+}-Ca^{2+} homo-exchange) via the Na^+/Ca^{2+} exchanger (for example, Blaustein & Santiago, 1977; DiPolo & Beaugé, 1987; Reeves & Poronnik, 1987). The ability of homo-exchange can be easily explained with consecutive transport models but not with simple simultaneous schemes. However, experimental data on ion competition (Blaustein & Santiago, 1977) and mathematical models appeared to be more consistent with a simultaneous mechanism (Hilgemann, 1988). Later it was pointed out that the competition experiments may be difficult to interpret unless the experiments were performed under zero-trans conditions (i.e., the concentration of the counter-substrate should be zero on the other side of the membrane; Läuger, 1987). Carried out under such conditions, the competition experiments again favored a consecutive scheme for the Na^+/Ca^{2+} exchanger (Khanansvhili, 1990; Li & Kimura, 1991).

More recently, experiments designed to obtain presteady state data on the Na^+/Ca^{2+} exchanger also supported a consecutive transport mechanism for the Na^+/Ca^{2+} exchanger (Hilgemann et al., 1991) or at least a mechanism that moves charge in more than one partial reaction step (Niggli & Lederer, 1991). In giant membrane patches the charge movements were initiated by sudden increments of the cytoplasmic Na^+ concentration in the absence of Ca^{2+} (Hilgemann et al., 1991). As had been predicted for consecutive exchangers, the apparent ion affinities of steady state I_{NaCa} increased as the counterion concentrations were decreased. In these experiments, the electrogenic step seemed to be at the extracellular end of the Na^+ translocation pathway (but *see* Matsuoka & Hilgemann, 1992) and the overall transport cycle was consistent with the concept of a consecutive transport mechanism (Hilgemann et al., 1991). Concentration jumps of $[Ca^{2+}]_i$ by means of the flash photolysis technique gave rise to transient membrane currents that

were consistent with a reaction scheme in which some fractional charge was moved during both, the Na^+ and Ca^{2+} translocation, again favoring consecutive movement of charge by the Na^+/Ca^{2+} exchange (Niggli & Lederer, 1991; Niggli & Lipp, 1994; Kappl & Hartung, 1996).

Turnover Rates

There are several estimates for Na^+/Ca^{2+} exchange turnover rates in the literature, but all numbers were derived rather indirectly. From the observed ion flux and the determined density of the Na^+/Ca^{2+} exchange protein a turnover rate of about 1,000 sec^{-1} has been estimated in sarcolemmal vesicles (Cheon & Reeves, 1988). From charge translocation experiments in isolated cells model dependent turnover rates for the Na^+/Ca^{2+} exchanger were also calculated and found to be less than 5,000 sec^{-1} (Hilgemann et al., 1991) or more than 2,500 sec^{-1} (Niggli & Lederer, 1991*b*) (but *see* Powell et al., 1993). Taken together, most data seem to suggest that the Na^+/Ca^{2+} exchanger is a transport protein with a high turnover rate, when compared to other membrane transporters (e.g., the Na^+-K^+ ATPase with a turnover rate of 60–200 sec^{-1}; Friedrich, Bamberg & Nagel, 1996).

Cloning of the Na^+/Ca^{2+} Exchanger

After several years of protein purification and isolation work (for example Soldati, Longoni & Carafoli, 1985; Cheon & Reeves, 1988; Philipson, Longoni & Ward, 1988; Vemuri et al., 1990) Na^+/Ca^{2+} exchange activity was expressed in *Xenopus* oocytes after injecting mRNA (Sigel et al., 1988; Longoni et al., 1988). Subsequent to this achievement, the canine cardiac Na^+/Ca^{2+} exchanger was cloned and sequenced (Nicoll et al., 1990). This event marked the onset of a new era of research on the Na^+/Ca^{2+} exchanger in heart cells. After combining the possibilities offered by molecular biology methods with the sophisticated biophysical techniques developed earlier we can expect to make rapid progress in our understanding of cellular and molecular Na^+/Ca^{2+} exchange function.

After the initial cloning of the Na^+/Ca^{2+} exchanger several isoforms were identified and sequenced in mammals (i.e., NCX1, NCX2, NCX3). The cardiac exchanger gene (coding for NCX1) exists as a single copy in the mammalian genome (Nicoll et al., 1990). The cardiac exchanger NCX1 is highly expressed in the heart and brain (Reuter & Porzig, 1995) but low levels are also found in different tissues including kidney, retina, skeletal and smooth muscle (Nicoll et al., 1990; Komuro et al., 1992; Kofuji et al., 1992; Kofuji, Lederer & Schulze, 1994; Low, Kasir & Rahamimoff, 1993; Li et al., 1994). NCX2 and NCX3 are expressed primarily in brain and skeletal muscle. The amino acid sequences of these isoforms are about 68–75% identical to each other (Li et al., 1994; Nicoll et al., 1996*a*). Several splice variants of NCX1 and NCX3 have been described, each exhibiting a specific tissue distribution (Kofuji et al., 1994; Quednau, Nicoll & Philipson, 1997). At present, the functional significance of the different isoforms is not yet understood since they all seem to behave similarly in several functional tests (Link et al., 1998).

Topology and Mutagenesis Studies

In functional expression studies several systems have been used successfully for the transient or stable expression of the Na^+/Ca^{2+} exchanger proteins including CHO cells (Condrescu et al., 1995), *Xenopus* oocytes (Nicoll et al., 1990), the BHK-cell line (Nicoll et al., 1996*a*), HeLa cells (Low et al., 1993.), HEK293 cells (Kofuji et al., 1992.) and Sf9 insect cells (Li et al., 1992, Egger et al., 1999*a*,*b*). In addition, electrogenic properties of the cloned transporter were demonstrated after reconstitution of the exchanger protein into phospholipid membranes (Low et al., 1994). In an animal model, the canine Na^+/Ca^{2+} exchanger was overexpressed in transgenic mice allowing functional studies in both isolated cells and in the intact cardiovascular system (Adachi-Akahane et al., 1997).

The NCX1 cDNA encodes a protein composed of 970 amino acids with a molecular mass of 120 kDa (Nicoll et al., 1990). Hydrophobicity analysis of the primary sequence suggested 11 transmembrane regions with a large hydrophilic region between segments 5 and 6 (loop f) (Nicoll et al., 1990; Doering et al., 1998, *see* Fig. 6). Ion exchanger and transport functions are associated with the transmembrane segments, whereas exchanger regulation appears to be mediated by the large cytoplasmic domain (loop f). The role of this large (520 amino acids) hydrophilic domain in exchanger regulation was examined by deletion mutagenesis (Matsuoka et al., 1993). For example, in Na^+/Ca^{2+} mutants in which the major part of the cytoplasmic domain had been deleted, both Na^+-dependent inactivation and Ca^{2+}-induced activation were found to be absent, whereas the ion transport mediated by the exchanger remained intact (Matsuoka et al., 1993).

The high affinity catalytic Ca^{2+} binding site (*see above*) has also been localized to a part of this loop (Levitsky, Nicoll & Philipson, 1994) and mutations in this region alter both Ca^{2+} binding and Ca^{2+} regulation of the exchange current (Matsuoka et al., 1995). For another segment of this loop, the positively charged endogenous exchange inhibitory peptide (XIP) region, an autoregulatory role in exchanger function has been suggested (Li et al., 1991; *see also* XIP blocker). This region is comprised of 20 amino acids at the amino ter-

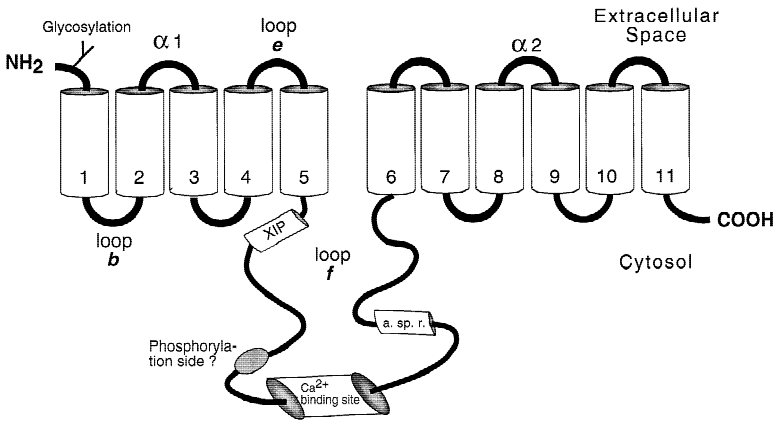

Fig. 6. Schematic drawing of the proposed membrane topology of the Na^+/Ca^{2+} exchanger (NCX1) based on hydropathy analysis. The intracellular loop (f) contains the regulatory Ca^{2+} binding site and the region for Na^+-dependent inactivation (endogenous XIP region). Between XIP and the Ca^{2+} binding region a potential protein kinase A phosphorylation site was identified. The transmembrane regions α-repeat 1 and α-repeat 2 are involved in ion translocation. Alternative splicing region (a.sp.r.; modified from Doering et al. 1998)

minus of the loop following the fifth transmembrane segment. Only the first 14 residues of the endogenous XIP are highly conserved. Recently, mutagenesis studies indicated that the endogenous XIP region located in the f loop is involved in movement of the exchanger into and out of the Na^+-induced inactivated state (*see* Na^+-dependent inactivation) and in the regulation by Ca^{2+} (Matsuoka et al., 1997).

An additional N-terminal hydrophobic domain (not included in Fig. 6) was shown to be a cleavable signal peptide targeting the protein to the cell membrane. Nevertheless, neither the presence nor the cleavage of the signal peptide is required for functional assembly of the exchanger protein in the membrane (Sahin-Toth et al., 1995). The extracellular location of the amino terminus of loop e (between segments 4 and 5) and the intracellular location of loop f have been verified experimentally (Hryshko et al., 1993; Porzig & Gutknecht, 1993; Sahin-Toth et al., 1995). With cysteine mutagenesis and sulfhydryl modification experiments it was recently confirmed that the loop connecting transmembrane segments 1 and 2 (loop b) is located on the cytoplasmic side (Doering et al., 1998). Mutations in this loop revealed that the cytoplasmic end of the transmembrane segment 2 may be involved in ion translocation and in regulation by cytoplasmic factors. Interestingly, other nearby mutations increased the affinity of the exchanger for cytoplasmic Na^+ and also produced a significant Li^+ transport capacity. In its native state, the Na^+/Ca^{2+} exchanger can discriminate between Na^+ and Li^+ and does not transport Li^+. It was proposed that the region at the interface of cytoplasmic loop b and transmembrane segment 2 is important in Na^+ transport and also in regulation by cytoplasmic Na^+ (Doering et al., 1998). An additional interesting domain in the large intracellular loop f is the variable region of NCX1 downstream of the regulatory Ca^{2+} binding site where alternative splicing occurs (Kofuji et al., 1994). Alternative splicing of a single NCX1 gene can produce 32 NCX1 isoforms.

Despite significant progress in our understanding of structure-function relationships, the molecular details of ion transport during a Na^+/Ca^{2+} exchange cycle and the 3-dimensional structure of the protein remain unknown. Furthermore, the molecular organization and self-association of the NCX1 exchanger protein in the plasma membrane has not yet been addressed experimentally. Based on crosslinking and hydrodynamic studies the retinal Na^+/Ca^{2+}-K exchanger present in the rod outer segments was proposed to exist as a homo-dimer (Schwarzer et al., 1997) in its natural environment. However, this was not experimentally verified *in situ.* Many transport proteins form oligomers, but it is not clear whether and how the Na^+/Ca^{2+} exchanger undergoes oligomerization and how this would affect the function.

Cellular Localization

In isolated cardiac ventricular myocytes the Na^+/Ca^{2+} exchanger can be immunolocalized in all membranes facing the extracellular space (Kieval et al., 1992; Frank et al., 1992). This distribution and arrangement provides an efficient Ca^{2+} extrusion from the entire cytoplasm. Confocal thin-section imaging revealed a regular grid of discrete foci of fluorescence (aligned with the Z-line), which represent Na^+/Ca^{2+} exchanger with an apparent high density in cardiac T-tubules (Kieval et al., 1992; Frank et al., 1996). The cardiac Na^+/Ca^{2+} exchanger binds ankyrin with high affinity. This interaction may be important for localizing the Na^+/Ca^{2+} exchanger to specific domains of the sarcolemma. Myocardial ankyrin could be localized to both surface and T-tubular sarcolemma. (Li et al., 1993). The higher density of exchanger molecules observed in the intercalated disc may reflect the folded character of this region and not necessarily a higher density. Ultrastructural apposition and functional access of the Na^+/Ca^{2+} exchanger molecules to the SR Ca^{2+} release sites (ryanodine receptors; RyRs) is presently investigated (Frank et al., 1996; Adachi-

Akahane, Cleemann & Morad, 1996; Adachi-Akahane et al., 1997).

Regulation

The cardiac sarcolemmal Na^+/Ca^{2+} exchanger has been observed to be regulated by several mechanisms in different experimental settings. However, for most regulatory mechanisms so far observed in vitro, it remains unclear whether and to which degree they are relevant under physiological or pathological conditions.

Na^+-Dependent Inactivation

An inactivation of the Na^+/Ca^{2+} exchange current by $[Na^+]_i$ was initially observed in giant patch experiments because this was the first technique allowing rapid changes of the Na^+ concentration facing the intracellular side of the transporter (Hilgemann, 1989). The Na^+-dependent inactivation was evident as a partial decay of the Na^+/Ca^{2+} outward exchange current within 1 sec after application of Na^+. The K_d for the Na^+-dependent inactivation was similar to the K_d for transport, suggesting that a significant fraction of the exchanger molecules might be in an inactivated state under physiological conditions. But Na^+-dependent inactivation was only observed when the sarcolemma was not pre-exposed to ATP (*see below* for the effects of ATP) and was removed when the cytoplasmic side was exposed to chymotrypsin (Hilgemann, 1989). From these results it was concluded that the large hydrophilic cytoplasmic loop f of the exchanger molecule may be essential for Na^+-dependent regulation since it would be likely to be cleaved by proteases. This view was later supported with deletion mutants as outlined above. Since intact myocytes normally contain millimolar cytosolic ATP concentrations, it was not clear whether Na^+-dependent inactivation would be present in intact cells. However, a study performed in freshly isolated ventricular cells suggested some role for $[Na^+]_i$ in Na^+/Ca^{2+} exchange regulation (Matsuoka & Hilgemann, 1994).

Ca^{2+}-Dependent Regulation

During the Ca^{2+}-dependent regulation (probably corresponding to the catalytic effect mentioned above) intracellular Ca^{2+} binds to a high affinity binding site that is distinct from the Ca^{2+} transport site (Hilgemann, 1990). The Ca^{2+} affinity of this site appears to depend on the experimental conditions. In the giant patch it is around 2 μM while in intact cells the Na^+/Ca^{2+} exchanger was already completely Ca^{2+} activated at the normal resting $[Ca^{2+}]_i$ (K_d for Ca^{2+} regulation 22 nM; Miura & Kimura, 1989). Taken together, it is still not clear whether $[Ca^{2+}]_i$ plays a regulatory role in vivo where $[Ca^{2+}]_i$ varies between 100 nM and more than 1 μM.

Intracellular ATP

Already early studies revealed that the Na^+/Ca^{2+} exchanger is stimulated by cytoplasmic ATP. Although no ATP is required to drive the exchange, ATP increased the affinity of the exchanger for both external Na^+ and internal Ca^{2+}, stimulating the Na^+-dependent Ca^{2+} efflux (Blaustein & Santiago, 1977). The exchanger was shown to have a high affinity to ATP with a half maximal activation at about 200 μM in squid axons (Dipolo & Baugé, 1987). In the giant patch, a stimulatory effect of ATP was also observed. This ATP-dependent stimulation of the Na^+/Ca^{2+} exchanger has a EC_{50} of ≈5 mM for ATP and may therefore reflect a mechanism different from the regulation in squid axon (Collins, Somlyo & Hilgemann, 1992).

A variety of mechanisms by which ATP might exert its action on the Na^+/Ca^{2+} exchange have been considered (*see* Hilgemann, 1997 for a recent review): (i) phosphorylation of the Na^+/Ca^{2+} exchanger or an associated protein by ATP-dependent protein kinases (Caroni & Carafoli, 1983; Iwamoto et al., 1996*a*); (ii) changes of the lipid environment resulting from the activation of phospholipid translocases ("flippases"; Hilgemann & Collins, 1992); (iii) ATP-dependent cytoskeletal processes (Li et al., 1993); (iv) a modulation of the Na^+/Ca^{2+} exchange activity by phosphatidylinositol-4,5-biphosphate (PIP_2; Hilgemann & Ball, 1996); (v) a direct effect of ATP on the Na^+/Ca^{2+} exchanger (Iwata et al., 1996).

The ATP-dependent stimulation of I_{NaCa} in the giant patch has been analyzed in some detail. Many tests for the involvement of protein kinases in the regulation of mammalian cardiac Na^+/Ca^{2+} exchangers by ATP were negative (for example Collins et al., 1992). However, ATP-dependent phosphorylation appears to be an important regulatory mechanism of the Na^+/Ca^{2+} exchanger in the squid axon (Dipolo & Beaugé, 1994*a*). Indeed, the recently cloned NCX-SQ1 has a potential protein kinase C phosphorylation site that is unique among all known exchanger sequences (He et al., 1998). In the giant patch, no clear evidence was obtained for a cytoskeletal involvement, but in this preparation the cytoskeleton is likely to be disrupted. It is established that the composition of the lipids in the plasma membrane has a dramatic effect on the Na^+/Ca^{2+} exchange activity, possibly resulting from lipid-protein interactions in the XIP region or from changes of local charges (Philipson & Ward, 1985; Collins & Hilgemann, 1993). But the physiological role of the phospholipids and changes of their properties mediated by ATP is not yet clear (Hilgeman & Collins, 1992b). Recently, an additional mechanism was proposed by which ATP could exert an effect

on the Na^+/Ca^{2+} exchanger. In giant patch experiments ATP appeared to act by stimulating lipid kinases generating PIP_2 from phosphatidylinositol (PI). Under these conditions, PIP_2 was observed to modulate both K_{ATP} channels and the Na^+/Ca^{2+} exchanger (Hilgemann & Ball, 1997).

In the Na^+/Ca^{2+} exchanger sequence of the frog a putative ATP binding site has been identified (Iwata et al., 1996). At present, it is not yet known whether ATP binds to this site and whether ATP binding results in allosteric effects. Interestingly, it was reported that in frog ventricular myocytes activation of the β-receptor/adenylate-cyclase/cAMP-dependent cascade results in suppression of transmembrane Ca^{2+} transport via the Na^+/Ca^{2+} exchanger (Fan, Shuba & Morad, 1996; Shuba et al., 1998).

pH

Changes of pH are known to inhibit or stimulate Ca^{2+} transport mediated by the Na^+/Ca^{2+} exchanger in cardiac sarcolemmal vesicles (Philipson et al., 1982; Khananshvili & Weilmaslansky, 1994). Although the intracellular pH is normally tightly controlled, some pathological cardiac conditions may result in cytoplasmic acidification (e.g., hypoxia, ischemia). In giant patch preparations the Na^+/Ca^{2+} exchanger was found to be very sensitive to changes of intracellular and extracellular pH (Doering & Lederer, 1993; Doering, Eisner & Lederer, 1996). A stepwise reduction in cytoplasmic pH from (7.2–6.4) produced a biphasic but monotonic decrease in Na^+/Ca^{2+} exchange current. Alkalinization of cytoplasmic pH (7.2–8.0) caused a large, biphasic increase in Na^+/Ca^{2+} exchange current, indicating that the protonation state of specific residues of the Na^+/Ca^{2+} exchanger or associated components are critical for the exchange function. Partial proteolysis with chymotrypsin abolished the proton sensitivity. Thus, the intracellular loop which contains regulatory sites for Na^+ and Ca^{2+} is likely to contain pH-sensitive residues responsible for the pH effects. Since the Na^+/Ca^{2+} exchanger is sensitive to the pH changes over a large range, it is assumed that several different residues affect the Na^+/Ca^{2+} exchanger at multiple sites and via several mechanisms. This view was also supported by the observation of a rapid and slow component of proton block at low pH (Doering & Lederer, 1993). The slowly developing block was dependent on the presence of Na^+ on the intracellular side and may be related to the Na^+-dependent inactivation discussed above (Doering & Lederer, 1994). Although we have learned quite a bit about proton effects on the Na^+/Ca^{2+} exchanger more details are necessary to appreciate the role of pH modulation in normal cardiac function and disease.

XIP

A polypeptide (exchange inhibitory peptide or XIP) corresponding to 20 amino acids of the loop f was synthesized and turned out to be a relatively potent inhibitor of the Na^+/Ca^{2+} exchanger (IC_{50} = 0.1–1.5 μM; (Li et al., 1991; Chin et al., 1993). Compared to available pharmacological tools, this blocker appears to be more specific (but *see* Enyedi & Penniston, 1993) and offers an alternative approach to investigate the cellular and molecular function of the Na^+/Ca^{2+} exchanger. At present, the precise mechanism by which XIP inhibits the exchanger is not yet understood on the molecular level (Xu, Gatto & Milanick, 1996; Shannon, Hale & Milanick, 1994). Charged residues of XIP as well as lipid-protein interactions seem to be important for the inhibitory effect (Xu et al., 1996). It has also been reported that XIP is a more potent inhibitor of the Na^+-Na^+ homo-exchange than of the Ca^{2+}-Ca^{2+} exchange mode. Using site-directed mutagenesis it was found that the XIP region plays a central role both in Na^+-dependent inactivation and Ca^{2+}-dependent regulation of the Na^+/Ca^{2+} exchanger (Matsuoka et al., 1997).

In several experiments XIP has been used to inhibit outward I_{NaCa} (Chin et al., 1993) and to test whether Ca^{2+} influx via reverse Na^+/Ca^{2+} exchange can trigger SR Ca^{2+} release and contractions in guinea pig ventricular myocytes (Bouchard et al., 1993; Kohmoto, Levi & Bridge, 1994).

Lipid-Protein Interactions

It has been determined in several experiments that the phospholipid composition of the membrane affects the biophysical properties of many membrane channels and transporters. An early observation was that optimal Na^+/Ca^{2+} exchange activity requires the presence of certain anionic phospholipids. In addition, incorporation of cholesterol greatly facilitated Na^+/Ca^{2+} exchange activity (Vemuri & Philipson, 1988). Various changes in the lipid environment of the Na^+/Ca^{2+} exchanger were found to inhibit or stimulate the protein (Hale et al., 1988, Behrson, Philipson & Weiss, 1991; Collins & Hilgemann, 1993). Since some of these lipid modifications can result from the activation of cellular signaling pathways (e.g., activation of phospholipases) or arise from pathophysiological conditions (e.g., ischemia, Corr & Cain, 1979), changes of the lipid environment may be more important for the regulation of Na^+/Ca^{2+} exchange than previously thought.

Phosphorylation

NCX1, NCX2 and NCX3 contain several consensus sites for phosphorylation by different kinases. The location of

the phosphorylation sites in the XIP region (NCX2 and NCX3) and in the loop b (NCX3) render them potentially interesting for regulation (Nicoll et al., 1996*a*,*b*). Interestingly, NCX1 does not have a phosphorylation site in the XIP region (Nicoll et al., 1996*a*). The role of protein phosphorylation in the regulation of different Na^+/Ca^{2+} exchangers is discussed in the section on intracellular ATP (*see above*).

Pharmacology

Although no specific pharmacological blocker of the Na^+/Ca^{2+} exchange is available, a variety of ions and compounds are known to have inhibitory effects. We compiled a list of inhibitors that are either physiologically relevant or are commonly used in experiments on the Na^+/Ca^{2+} exchanger (*see* Table).

Tri- and Divalent Cations

Heavy metal ions that interact at different bindings sites of the exchanger inhibit and/or compete with transport of Ca^{2+} and/or Na^+, e.g., La^{3+}, Ni^{2+}, Cd^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}. Some of these ions act competitively and are also substrates for the transport (e.g., Ba^{2+} and Sr^{2+}) (Kimura et al., 1987; Niggli, 1989; Condrescu et al., 1997). The use of the most potent inorganic inhibitors La^{3+} and Ni^{2+} is limited because both also inhibit other membrane currents (*see also* section on the parmacological isolation of I_{NaCa}). As already mentioned the extracellular application of Ni^{2+} can be used to specifically inhibit I_{NaCa}, but only under conditions where other ionic currents affected by Ni^{2+} were already eliminated by more specific blockers (Kimura et al., 1987). However, the mechanism by which these inhibitors block the Na^+/Ca^{2+} exchanger is poorly understood. For example, even though Ni^{2+} completely inhibits the Na^+/Ca^{2+} exchange current, it is at the same time rapidly transported by the exchanger, presumably with an electroneutral stoichiometry (Egger et al., 1999*a*,*b*).

Organic Inhibitors

Several organic inhibitors for the Na^+/Ca^{2+} exchanger have also been identified, including amiloride and its derivatives (e.g., 3′,4′-dichlorobenzamil; DCB), bepridil, quinacrine, dodecylamine, KB-R7943 (formerly No. 7943). Unfortunately, none of these blockers was found to be very specific for the Na^+/Ca^{2+} exchanger because other channels and transporters (e.g., L-Type Ca^{2+} channels) were affected in the same range of concentrations (Bielefeld et al., 1986). Interestingly, several organic inhibitors were reported to block some transport modes of the Na^+/Ca^{2+} exchanger more than other modes. This property might be an important feature for the future development of Na^+/Ca^{2+} exchange inhibitors with different therapeutical profiles (Reeves, 1989). For example, amiloride derivatives like DCB block both Na^+-dependent Ca^{2+} uptake and Na^+-dependent Ca^{2+} efflux: interestingly, Na^+-Na^+ exchange is inhibited in a competitive fashion while Ca^{2+}-Ca^{2+} exchange is still active at physiological K^+ concentrations (Slaughter et al., 1988). At low concentrations, amiloride analogues are interacting preferentially with a site that is exclusive for Na^+, while at higher concentration they also interact at a site that is common for Na^+ and Ca^{2+}, suggesting a complex interaction between inhibitor and exchanger (Slaughter et al., 1988). DCB preferentially inhibits the inward rather than the outward Na^+/Ca^{2+} exchange current (Lipp & Pott, 1988*b;* Iwamoto, Watano & Shigekawa, 1996*b*).

Recently, the isothiourea derivative KB-R7943 (No. 7943) has been reported to fairly selectively inhibit the Na^+/Ca^{2+} exchanger (NCX1) (Watano et al., 1996; Iwamoto et al., 1996*b;* Watano & Kimura, 1998). Block of the Na^+/Ca^{2+} exchange current by this compound was analyzed in single guinea pig myocytes. It blocked the outward and the inward I_{NaCa} reversibly and in a concentration-dependent manner ($IC_{50} \approx 0.32$ μM for outward and ≈17 μM for inward I_{NaCa}, respectively). This was in contrast to DCB which suppressed the inward Na^+/Ca^{2+} exchange current with an IC_{50} of 17 μM, but did not affect the outward exchange current under these conditions. Unfortunately, the I_{Na}, I_{Ca} and the inward rectifier K^+ current were also inhibited by KB-R7943 with an IC_{50} of approximately 14 μM, 8 μM and 7 μM, respectively (Watano et al., 1996). Inhibition of the Na^+/Ca^{2+} exchanger was shown to be noncompetitive with respect to Ca^{2+} and Na^+ both in cells and sarcolemmal vesicles. These results suggest that the blocker primarily acts on external exchanger site(s) other than the transport sites, although it seems to be able to inhibit the exchanger from both sides of the plasma membrane (Iwamoto et al., 1996*b*). Dodecylamine, quinacrine and DCB, well known Na^+/Ca^{2+} exchange inhibitors in vesicles, also block creep currents and even at concentrations well below those required to block Na^+-dependent Ca^{2+} uptake. The sensitivity of creep currents to these compounds is consistent with the hypothesis that creep currents may represent the electrogenic activity of the Na^+/Ca^{2+} exchanger (Bielefeld et al., 1986).

Antisense Oligonucleotides

Making use of the available sequence information of the Na^+/Ca^{2+} exchanger an alternative approach was successfully applied to suppress the Na^+/Ca^{2+} exchanger in cultured cardiac myocytes (Lipp, Schwaller & Niggli, 1995, Takahashi et al., 1995) as well as other prepara-

Table 1. Unspecific inhibition of the Na^+/Ca^{2+} exchange activity

Blocker	Inhibition of Na^+/Ca^{2+} exchange	Method	Parameter	Preparation	Reference
La^{3+}	3 mM ≈ 100%	Tonic contraction	Force	FA[a]	Horackova & Vassort, 1979
	50 μM ≈ 80–90%	Whole cell	$I_{creep(in\&out)}$	FA	Hume & Uehara, 1987
	IC_{50} = 400 μM	Ca, Na-flux	Ca_o^{2+}-dependent-Na^+ efflux	Barnacle Muscle	Rasgado-Flores et al., 1989
	500 μM ≈ 100%	Whole cell	I_{NaCa}	GPV[b]	Kimura et al., 1987
Cd^{2+}	IC_{50} = 50 μM	Ca, Na-flux	Na_i^+-dependent-Ca^{2+} uptake	RV[c]	Trosper & Philipson, 1983
	K_i = 320 μM	Whole cell	I_{NaCa}	RV	Hobai et al., 1997
	1 mM ≈ 100%	Whole cell	I_{NaCa}	GPV	Kimura et al., 1987
Co^{2+}	1 mM ≈ 100%	Excised giant patch	I_{NaCa}	GPV	Hilgemann, 1989
Mn^{2+}	IC_{50} = 250 μM	Ca, Na-flux	Na_i^+-dependent-Ca^{2+} uptake	CSVesicles[d]	Trosper & Philipson, 1983
	1 mM ≈ 100%	Whole cell	I_{NaCa}	GPV	Kimura et al., 1987
Ni^{2+}	1.0–5.0 mM ≈ 70–100%	Whole cell	I_{NaCa}	GPV	Kimura et al., 1987, Beukelmann, 1989
	IC_{50} = 200 μM	Whole cell	I_{NaCa}	GPV	Levi, 1998[e]
	8 mM ≈ 100%	Whole cell flash photolysis	I_{NaCa}	GPV	Niggli & Lederer, 1993
Extracellular $[H^+]$	pH < 5.0 ≈ 80–100% pH > 10 ≈ 40–50%	Whole cell, flash photolysis	I_{NaCa}	GPV	Egger & Niggli, 1998
Intracellular $[H^+]$	pH < 6.0 ≈ 80–90%	Excised giant patch	I_{NaCa}	GPV	Doering & Lederer, 1993
	pH < 6.0 ≈ 90%	Ca-influx	Na_i^+-dependent-Ca^{2+} uptake	DSVesicles[e]	Philipson et al., 1984
Amiloride	3.0 mM ≈ 25%	Whole cell	I_{NaCa}	GPV	Kimura et al., 1987
	IC_{50} = 1 mM	Ca-flux	Na_o^+-dependent-Ca^{2+} uptake	GH[f]	Kaczorowski et al., 1985
Benzamil	K_i = 100 μM	Ca, Na-flux	Na_i^+-dependent-Ca^{2+} uptake	BSVesicles	Slaughter et al., 1988
	IC_{50} = 100 μM	Ca-flux	Na_o^+-dependent Ca^{2+} uptake	GH	Kaczorowski et al., 1985
D600[æ]	22 μM ≈ 65%	Whole cell	I_{NaCa}	GPV	Kimura et al., 1987
	2.2 μM ≈ 75%	Whole cell	Slow inward current	FA	Mentrard et al., 1984
DCB[#]	K_i = 20 μM	Ca-flux	Na_i^+-dependent-Ca^{2+} influx	BSVesicles[g]	Slaughter et al., 1988
	IC_{50} = 36 μM	Ca, Na-flux	Na_o^+-dependent-Ca^{2+} efflux	BSVesicles	Slaughter et al., 1988
	IC_{50} = 17 μM	Whole cell	I_{NaCa}	GPV	Watano et al., 1996
	K_i = 4 μM	Whole cell	$I_{creep(in\&out)}$	FA	Bielefeld et al., 1986
	IC_{50} = 17 μM	Force, Ca-influx	Na_i^+-dependent-Ca^{2+} uptake	GPSVesicles[h] PM[i]	Siegl et al., 1984
	IC_{50} = 30 μM	Whole cell	$I_{ti}^{\S}$	GPA[k]	Lipp & Pott, 1988
	100 μM ≈ 85–90%	Whole cell, flash photolysis	I_{NaCa}	GPV	Niggli & Lederer, 1991
Dodecylamin	K_i = 20 μM	Ca-flux	Na_i^+-dependent-Ca^{2+} uptake	DSVesicles	Philipson et al., 1984
	K_i = 3 μM	Whole cell	$I_{creep(inward)}$	FA	Bielefeld et al., 1986
FMRF-amide[Δ]	K_i = 1.5 μM	Ca-flux	Na_o^+-dependent-Ca^{2+} efflux	Squid axons	DiPolo & Beauge, 1994

Continued on next page

Table 1. *Continued*

Blocker	Inhibition of Na^+/Ca^{2+} exchange	Method	Parameter	Preparation	Reference
Quinacrine	1 mM ≈ 90%	Whole cell, flash photolysis	I_{NaCa}	GPV	Niggli & Lederer, 1993
	K_i = 10 μM	Whole cell	$I_{creep(inward)}$	FA	Bielefeld et al., 1986
Verapamil	K_i = 50 μM	Ca-flux	Na_o^+-dependent-Ca^{2+} uptake	BSVesicles	Kosnev et al., 1989
	IC_{50} = 50 μM 200 μM ≈ 66%	Ca-flux	Na_i^+-dependent-Ca^{2+} uptake	BSVesicles	Erdreich & Rahamimoff, 1984
XIP°	K_i = 0.1 μM	Excised giant patch	I_{NaCa}	RV	Li et al., 1991
	IC_{50} = 1.5 μM[&]	Ca-flux	Na_i^+-dependent-Ca^{2+} uptake	CSVesicles	Li et al., 1991
	IC_{50} = 3 μM	Ca-flux	Na_o^+-dependent-Ca^{2+} uptake	BSVesicles	Shannon et al., 1994
#7943* (KB-R7943)*	IC_{50} = 17 μM	Whole cell	Inward I_{NaCa}	GPV	Watano et al., 1996
	IC_{50} = 0.32 μM	Whole cell	Outward I_{NaCa}	GPV	Watano et al., 1996

[æ] methoxyverapamil (also block T-type Ca^{2+} channels); [#]3′,4′-dichlorobenzamil, *(2-[2-[4-{4-nitrobenzyloxy)phenyl]isothiourea-methanesulfonate; °exchanger inhibitory peptide; [Δ]molluscan cardioexcitatory tetrapeptide amide Phe-Met-Arg-Phe-NH_2; [§] transient inward current; [&] maximal inhibition 80%; [£]personal communication; *Preparations:* [a]frog atrial cells; [b]guinea pig ventricular cells; [c]rabbit ventricular cells; [d]canine sarcolemmal vesicles; [e]dog sarcolemmal vesicles; [f]GH_3 rat anterior pituitary cells; [g]bovine sarcolemmal vesicles; [h]guinea pig sarcolemma vesicle; [i] papillary muscle; [k]guinea pig atrial cells.

tions (Slodzinski, Juhaszova & Blaustein, 1995; Slodzinski & Blaustein, 1998*a*,*b*). An antisense oligonucleotide directed against 19 nucleotides in the 3′ nontranslated region of the Na^+/Ca^{2+} exchanger was synthesized (Lipp et al., 1995). The exposure of the myocytes to this antisense oligonucleotide for 24–48 hours completely inhibited the decay of Ca^{2+} transients induced by photolysis of caged Ca^{2+} in most cells (i.e., Ca^{2+} efflux via Na^+/Ca^{2+} exchanger). Increases in intracellular Ca^{2+} usually observed during superfusion with Na^+-free media were also abolished (i.e., the Ca^{2+} influx mode of the Na^+/Ca^{2+} exchanger). Antisense oligodeoxynucleotides may therefore represent another useful tool to investigate the cellular and molecular properties of the Na^+/Ca^{2+} exchanger (Schwaller et al., 1999).

Clinical Implications

Cardiac Hypertrophy and Failure

The role and alterations of the Na^+/Ca^{2+} exchanger in cardiac hypertrophy and failure remains controversial. Some of the controversial conclusions may arise from differences of the disease model system used. In addition, quite different techniques were employed and diverse parameters were determined in these studies. Furthermore, the Ca^{2+} signaling (and Na^+/Ca^{2+} exchange) may undergo a sequence of distinctive changes in the course of the progredient disease. Thus it is likely that diverse cellular systems important for Ca^{2+} signaling are altered in the hypertrophied and failing heart. Several groups have reported that the expression of the Na^+/Ca^{2+} exchanger is upregulated in heart cells with myocardial hypertrophy or heart failure (e.g., Kent et al., 1993; Flesch et al., 1996), an observation possibly related to the concomitant activation of the adrenergic system (Reinecke, Vetter & Drexler, 1997). It has been proposed that increased Ca^{2+} extrusion via the Na^+/Ca^{2+} exchanger may represent an adaptive mechanism and may partly compensate for the impaired SR Ca^{2+} re-uptake resulting from the downregulation of the SR Ca^{2+} pump (Studer et al., 1994). Overall, a prolongation of the cytosolic Ca^{2+} transients would nevertheless prevail because of the reduced Ca^{2+} uptake by the SR (Morgan, 1991; Studer et al., 1994). A prolongation of the Ca^{2+} transient was confirmed by experiments performed in myocytes isolated from pressure-overloaded hearts, but this functional study indicated that the Na^+/Ca^{2+} exchange activity was not significantly changed (Maier et al., 1998).

Interestingly, the number of Ca^{2+} channels and the Na^+/Ca^{2+} exchange activity was found to be reduced during early stages of heart failure induced by ligating the left ventricular artery in rat hearts. It may thus be possible that changes in Na^+/Ca^{2+} exchange and Ca^{2+} channels contribute towards decreasing Ca^{2+} influx and load, but only during early stages of heart failure (Dhalla et al., 1991). In another study, contractions of normal or increased amplitude were observed in myocytes isolated

from infarcted hearts, despite a decrease in peak Ca^{2+} currents. Since the Na^+/Ca^{2+} exchange current density was increased in these cells Ca^{2+} influx via the Na^+/Ca^{2+} exchanger was assumed to support the contractility that would otherwise be reduced (Litwin & Bridge, 1997).

Ischemia

Alterations in the Na^+/Ca^{2+} exchange activity in myocytes would be expected during ischemia (Meno, Jarmakani & Philipson, 1989; Tani, 1990). These effects mainly result from changes of the intracellular Na^+ concentration and pH (Wilde & Kléber, 1986). Modifications of $[Na^+]_i$ directly affect the electrochemical forces driving the Na^+/Ca^{2+} exchanger. Intra- and extracellular acidification are both known to reduce the Na^+/Ca^{2+} exchange transport rate (Doering & Lederer, 1993; Egger & Niggli, 1998), as outlined in the section on pH regulation above. The lipid environment surrounding the Na^+/Ca^{2+} exchanger may also be modified during ischemia with consequences for the transporter. The accumulation of lysophosphoglycerides has been implicated in some of these effects (Corr & Cain, 1979) and was shown to indeed influence the Na^+/Ca^{2+} exchange activity (Behrson et al., 1991). Other changes of the sarcolemmal lipid and fatty acid composition may also occur during ischemia and may have secondary effects on the Na^+/Ca^{2+} exchanger (Philipson & Ward, 1985; Bersohn et al., 1991; Collins et al., 1993).

Na^+/Ca^{2+} Exchange Inhibitors as Cardiotonic Agents

The potential therapeutic usefulness of Na^+/Ca^{2+} exchange inhibitors as cardiotonic agents was reviewed several years ago (Reeves, 1989; Ravens & Wettwer, 1989). Inhibition of the Ca^{2+} efflux mode or stimulation of the Ca^{2+} influx mode would be expected to exert a positive ionotropic effect, similar to cardiotonic steroids. Cardiotonic steroids are examples of drugs that interfere with the Na^+/Ca^{2+} exchange to increase cardiac force, albeit indirectly. These drugs are known to inhibit the sarcolemmal Na^+-K^+ ATPase leading to an increased intracellular Na^+ concentration (for review *see* Lee, 1985). The rise in $[Na^+]_i$ in turn results in a decreased driving force for the Ca^{2+} efflux via Na^+/Ca^{2+} exchanger and hence to an accumulation of intracellular Ca^{2+} and an increase of the SR Ca^{2+} load. Augmented SR Ca^{2+} release finally restores the contractile force but, as a side-effect, also causes a tendency for spontaneous SR Ca^{2+} release that may be responsible for arrhythmias (Trafford, O'Neill & Eisner, 1993). Nevertheless, the development of specific Na^+/Ca^{2+} exchange inhibitors is desirable. Besides their therapeutic potential such compounds would represent useful tools to investigate the cellular and molecular function of the Na^+/Ca^{2+} exchanger in vivo and in vitro.

Outlook

Present molecular biology has developed methods to modify the expression of genes in animals, to study the factors responsible for the tissue-specific expression and the regulation of expressed proteins. As these methods are applied to genes that code for the Na^+/Ca^{2+} exchanger, new insight into its control and regulation is to be expected. These techniques could be used to compensate, at least in part, for the incomplete and imperfect Na^+/Ca^{2+} exchange pharmacology. Currently, three strategies are commonly used: (i) overexpression of the Na^+/Ca^{2+} exchanger. A transgenic mouse overexpressing the Na^+/Ca^{2+} exchanger has been used to investigate several aspects of Na^+/Ca^{2+} exchange function during EC-coupling (Adachi-Akahane et al., 1997). (ii) the function of a gene product can be investigated by deleting the gene of interest (transgenic knockout systems). Several gene "knockout" mice exist with deletions in genes that are involved in Ca^{2+} signaling (Buck et al., 1997; Schwaller et al., 1997, Airaksinen et al., 1997). At present, a Na^+/Ca^{2+} exchange knockout mouse is not yet available. A modification of the basic knockout technology allows carrying out "conditional knockouts" that can be activated pharmacologically at any stage of development. This approach can be used when conventional knockout is lethal or if the induction of compensatory mechanisms are expected, which may be possible for the Na^+/Ca^{2+} exchanger. (iii) The functional expression of a gene product may be specifically suppressed with antisense oligonucleotides. This approach has already been applied in cultured cardiac myocytes (Lipp et al., 1995; Niggli, Schwaller & Lipp, 1996). However, antisense studies in adult cardiac myocytes are hampered by the slow onset of the antisense effect and the rapid de-differentiation of these cells in culture (Eppenberger et al., 1988).

The structure of the Na^+/Ca^{2+} exchanger has hardly been studied and very little is known about it. A specific goal for the future would thus be to obtain more information about the 3-dimensional structure of the Na^+/Ca^{2+} exchanger protein. The functional peculiarities of the exchanger isoforms expressed in various tissues are also not yet known. The question how the Na^+/Ca^{2+} exchanger achieves its tissue-specific function certainly deserves a significant research effort. Functional differences of the various isoforms are likely to exist and may provide a lead to the understanding of molecular and functional diversity of the Na^+/Ca^{2+} exchanger. At the center of interest in biophysical studies at the molecular level is the question of regulatory elements for intra- and extracellular ion binding and translocation and

the elucidation of structure-function relationships. In this context, it would also be important to know details about each biochemical reaction step of the transport cycle.

While several regulatory mechanisms of the Na^+/Ca^{2+} exchanger have been identified under highly artificial experimental conditions, it is not clear whether and by which signaling pathways the mammalian heart can regulate the Na^+/Ca^{2+} exchanger. Physiologically important regulatory mechanisms may include protein expression (Smith & Smith, 1995; Smith et al., 1995), phosphorylation as well as lipid-protein interactions. Experiments with transgenic animals may help to provide answers to some of these questions in the near future.

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