Sodium/Calcium Exchanger (NCX1) Macromolecular Complex*

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The sodium-calcium exchanger, NCX1, is a ubiquitously expressed membrane protein essential in calcium homeostasis for many cells including those in mammalian heart and brain. The function of NCX1 depends on subcellular (“local”) factors, the phosphorylation state of NCX1, and the subcellular location of NCX1 within the cell. Here we investigate the molecular organization of NCX1 within the cardiac myocyte. We show that NCX1 is dynamically phosphorylated by protein kinase A (PKA)-dependent phosphorylation in vitro. We also provide evidence that the regulation of this phosphorylation is attributed to the existence of an NCX1 macromolecular complex. Specifically, we show that the macromolecular complex includes both the catalytic and regulatory subunits of PKA. However, only the RI regulatory subunit is found in this macromolecular complex, not RII. Other critical regulatory enzymes are also associated with NCX1, including protein kinase C (PKC) and two serine/threonine protein phosphatases, PP1 and PP2A. Importantly, the protein kinase A-anchoring protein, mAKAP, is found and its presence in the macromolecular complex suggests that these regulatory enzymes are coordinately positioned to regulate NCX1 as has been found in diverse cells for a number of channel proteins. Dual immunocytochemical staining showed the colocalization of NCX1 protein with mAKAP and PKA-RI proteins in cardiomyocytes. Finally, leucine/isoleucine zipper motifs have been identified as possible sites of interaction. Our finding of an NCX1 macromolecular complex in heart suggests how NCX1 regulation is achieved in heart and other cells. The existence of the NCX1 macromolecular complex may also provide an explanation for recent controversial findings.

The Na\(^{+}/Ca\(^{2+}\)) exchanger, NCX1, is an integral membrane protein that is expressed in many tissues and is involved in cellular Ca\(^{2+}\) homeostasis (1, 2). The expression level of NCX1 is modulated during development (3, 4) and under pathological conditions (5–9). The Na\(^{+}/Ca\(^{2+}\)) exchanger activity has been shown to be affected by the ions that it transports (Na\(^{+}\) and Ca\(^{2+}\)) (10–13), by protons (14, 15), by phosphatidylinositol 4,5-bisphosphate in the membrane (16), and by exogenous agents including intracellular application of an inhibitor peptide (XIP) (17). However, regulation of NCX1 by PKA\(^{1}\) has remained controversial (18–23).

Early studies suggested that ATP-dependent regulation of NCX1 occurred in squid axons (24, 25) and in cardiac sarcoplasmic vesicles (26), but these studies did not distinguish between direct ATP binding and ATP-dependent phosphorylation. Several studies were designed to resolve this problem. Hilgemann and colleagues (11, 12, 27, 28) investigated the question by measuring NCX1 currents in giant excised patches. The work used two preparations, NCX1-expressing Xenopus oocytes (27) or cardiac myocytes expressing native NCX1 (11, 12, 28). These investigations found no functional change in cardiac NCX1 activity following application of PKA or protein kinase C (PKC) catalytic subunits to the intracellular side of the giant patch. Condrescu et al. (19) came to similar conclusions by examining the phosphorylation state of NCX1 in a heterologous expression system (19). Less direct investigations, however, suggested that PKA affected NCX1 function in heart (29, 30). The indirect nature of these studies in support of phosphorylation did not exclude the possibility that the measured changes were due to PKA phosphorylation of other proteins. The first unambiguous demonstration that phosphorylation affected NCX1 was provided by Iwamoto et al. (31). They showed that NCX1 from rat aorta smooth muscle cells is phosphorylated by PKC and is activated in response to growth factors (32). Further studies demonstrated that cardiac NCX1 is regulated by PKC phosphorylation (20). Additionally, Iwamoto et al. (20) provided evidence that the intracellular loop of NCX1 was phosphorylated by PKC and PKA. We were the first to show that PKA-dependent phosphorylation of NCX1 increased NCX1 activity both in Xenopus oocytes expressing cardiac NCX1 and in adult rat ventricular cardiomyocytes (33, 34). To determine how such divergent conclusions could arise, we undertook an investigation of the molecular organization of NCX1 in heart.

Regulation of NCX1 could arise through cytosolic enzymes bathing NCX1 or through local clustering of the regulatory proteins. The variable loss of cytosolic regulatory proteins during experiments on NCX1 phosphorylation could, in principle, account for the divergent findings. Alternatively, if NCX1 was part of a macromolecular complex that included other regulatory proteins, differences in experimental results may reflect the different levels of expression and activity of associated proteins under different experimental conditions. Recently, local signaling complexes have been shown to regulate ion channels similar to the l-type Ca\(^{2+}\) channel (35) and specific K\(^{+}\) channels (36) and cardiac ryanodine receptors (RyR2) (37). These complexes are composed of kinases, phosphatases, and kinase-anchoring proteins (AKAPs) and regulate activation state, sub-

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1 The work was supported by grants from the American Heart Association and NHLBI and NIA, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: PKA, protein kinase A; PKC, protein kinase C; RyR, ryanodine receptors, mAKAP, muscle protein kinase A-anchoring protein; AKAP, A-kinase-anchoring proteins; TM, transmembrane; LZ, leucine/isoleucine-zipper.
strate specificity, and subcellular localization. Recently, it was shown that the RyR2 could be more rapidly de-phosphorylated by the phosphatases of the macromolecular cluster than the kinases phosphorylated RyR2 (38). This recent observation and the large number of Ca\(^2+\)-regulatory proteins associated with macromolecular complexes that regulate function suggested that this hypothesis is particularly appealing for NCX1. The experiments that we present below examine the macromolecular complex hypothesis for NCX1.

Recently, we have shown that phosphorylation of NCX1 is induced by \(\beta\)-adrenergic stimulation in pig heart resulting in increased NCX1 exchange current (I\(_{\text{NCX}}\)). More importantly, we found that NCX1 is hyperphosphorylated in pig model of heart failure but \(\beta\)-adrenergic response is attenuated in heart failure (39). These findings underscore the significance of NCX1 phosphorylation in pathological heart, and we have investigated the regulation of NCX1 phosphorylation in this paper. Preliminary findings of this work were presented in abstract form previously (40).

**EXPERIMENTAL PROCEDURES**

**Preparation of Heart Extract**

F344 strain rats (NIA, National Institutes of Health) were sacrificed by injecting with pentobarbitral, and the hearts were removed immediately. After rinsing in ice-cold phosphate buffered saline, the ventricles were frozen in liquid nitrogen. The ventricles were pulverized in liquid nitrogen using a mortar and pestle and then homogenized in glass homogenizer (Eberbach, Ann Arbor, MI) for 20 min over ice. During homogenization, the extraction buffer composed of 50 mM NaCl, 3 mM KCl, 25 mM sodium pyrophosphate, 10 mM ATP, 5 mM EDTA (pH 7.4), and protease inhibitors (50 \(\mu\)g/ml phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, 1 \(\mu\)g peptatin, 2 \(\mu\)g/ml leupeptin, 1000 units/ml aprotinin, and 1 mM 1,10-phenanthroline) was added gradually. Undissolved cell fragments were removed by centrifugation at 3000 rpm for 15 min at 4 °C. The turbid supernatant was homogenized again over ice and centrifuged at 5000 rpm and at 4 °C (Eppendorf centrifuge 5415C). The supernatant was concentrated 3-fold using Micro-50 (Millipore) at 4 °C. and the concentrate was used either for electrophoresis as cardiac lysate or for immunoprecipitation for NCX1 macromolecular complex.

**Immunoprecipitation and in Vitro Phosphorylation**

The cardiac lysate was incubated with NCX polyclonal antibody \(\alpha11-13\) (Swaet, Bellinzona, Switzerland) overnight, and the antigen-antibody complex was precipitated by mixing with protein A-Sepharose beads (Sigma). To identify the full-length mAKAP, immunoprecipitation was done using monoclonal NCX1 antibody (R3F1, Swant). The complex was washed in buffer containing 150 mM NaCl, 6 mM EDTA, 50 mM Tris (pH 7.4), 0.1% Triton X-100, and 0.02% SDS. Phosphorylation was performed by incubation of the pellet with 1 \(\mu\)g of the catalytic subunit of PKA (PKA-CS reconstituted in 5 mM dithiothreitol) and 10 \(\mu\)Ci of \(^{32}\)P-ATP (3000 Ci/mmol) in phosphorylation buffer (25 mM Hepes, 5 mM MgCl\(_2\), 5 mM EGTA, and 0.2% Triton X-100 (pH 7.4)) for 10 min at 37 °C. In experiments with inhibitor, PKA inhibitor (Sigma) was included along with PKA catalytic subunit. The reaction was stopped by washing with 1 ml of RIA buffer (50 mM sodium phosphate buffer (pH 7.4), 50 mM KP, 75 mM NaCl, 2.5 mM EDTA, 0.01% NaN\(_3\), and 25 mM Tris (pH 7.4)) (41). Samples were heated to 75 °C for 3 min in gel loading buffer containing 100 mM dithiothreitol and analyzed using 8% polyacrylamide gels. The proteins from the gel were transferred to nitrocellulose membrane (Amersham Biosciences) and exposed to Kodak X-Omnat AR at –80 °C.

**Western Blotting and Antibodies**

After the immunoprecipitation with NCX1 antibodies and protein A-Sepharose beads, the protein sample was treated as described above. After electrophoresis, the proteins were transferred to nitrocellulose membrane and the primary antibodies were added. The following antibodies were used for immunoblotting: rabbit polyclonal to NCX1 (\(\alpha11-13\), Swant), mouse monoclonal to PKA catalytic subunit (clone 5D); to PKA RI subunit (Clone 18), to PKC (Clone MC5), and to PPI (clone 24) (BD Biosciences); rabbit polyclonal to mA\(K\)AP (Upstate Biotechnology); and mouse monoclonal to PP2A (Clone 6P9) (CRP Inc.). HRP-conjugated appropriate secondary antibodies (Jackson Immunoresearch Lab-

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**FIG. 1. Phosphorylation of cardiac NCX1 protein. Left panel,** after immunoprecipitation of the NCX1 protein from rat heart, three bands (120, 140, and 160 kDa) were identified using Western blotting and the NCX1 antibody. **Middle panel,** the immunoprecipitated NCX1 protein was phosphorylated in vitro using PKA catalytic subunit and \(^{32}\)P-ATP. The bands at 120 and 160 kDa were highly phosphorylated (right arrowheads). **Right panel,** during in vitro phosphorylation, PKA inhibitory peptide, PKI, was included and prevented NCX1 phosphorylation. The protein marker at 97 kDa is indicated by the arrow on the left. West, Western blot; Autorad, autoradiograph.

**RESULTS**

**In Vitro Phosphorylation of NCX1 in Heart**

Increased NCX1 activity in rat ventricular cardiomyocytes following PKA activation (30, 33) could be attributed to the direct effect on NCX1 protein or could be due to PKA activation of another target protein. To investigate these alternatives, we examined the phosphorylation state of NCX1. Total cardiac protein was prepared, and NCX1 protein was immunoprecipitated with NCX1 antibody. A Western blot permitted identification of NCX1 protein as three bands at 120, 140, and 160 kDa (Fig. 1, left panel). These bands are characteristically observed for NCX1 protein (42). When the immunoprecipitated cardiac NCX1 was phosphorylated in vitro using catalytic subunit of PKA and \(^{32}\)P-ATP, the bands at 120 and 160 kDa were clearly phosphorylated (Fig. 1, middle panel). Inclusion of an inhibitor of PKA (PKI, the PKA inhibitory peptide) during the phosphorylation reaction prevented the labeling of these proteins, indicating the specificity of PKA on rat cardiac NCX1 (Fig. 1, right panel). This experiment demonstrates that cardiac NCX1 is phosphorylated by PKA in support of the findings of Iwamoto et al. (20).

**Protein Kinases and NCX1**

To examine the possibility that NCX1 associates with relevant kinases, NCX1 was immunoprecipitated and immunoblotting was performed. The PKA holoenzyme is a kinase composed of two identical catalytic subunits and two identical regulatory subunits (43). We show that the components of the PKA holoenzyme, both the catalytic and regulatory subunit RI, are recognized in the NCX1 immunoprecipitate by the antibodies specific for those proteins (Fig. 2A). The other PKA regulatory subunit, RII, was not detected in the macromolecular complex. This indicates that only RI is associated with NCX1. All of the
PKA components (including RI) were found in the cardiac lysate (Fig. 2, AII). The presence of both catalytic and regulatory subunits in the cardiac lysate shows that PKA components are also found in the soluble components of the cell as has been reported previously (44). Using the same immunoprecipitation strategy, we investigated whether or not PKC is associated with NCX1. As shown in Fig. 2B, a sharp single band is present on the immunoblot using a pan-PKC antibody.

Phosphatases and NCX1

The absence of measured phosphorylation of NCX1 following PKA activation in some studies could be compatible with our results if there was a difference in the rate of dephosphorylation in the various studies. Conditions that may lead to such differences could occur if phosphatases colocalized with NCX1 as has been shown for RyR2 (38). Fig. 3 examines this by immunoblot analysis with antibodies to PP1 and PP2A as shown in Fig. 3, A and B, respectively. We found that the serine/threonine phosphatases PP1 and PP2A were precipitated along with the NCX1. These phosphatases were also readily detected in the cardiac lysate.

AKAP and NCX1

Targeting of kinases and phosphatases to specific proteins has been described extensively by Scott and co-workers (45, 46). These studies have identified a family of “a kinase anchoring proteins” or AKAPs that serves as scaffolding proteins for this function. To explore the possibility that an AKAP may be associated with NCX1, we probed immunoblots with antibodies against six of the major AKAPs. Fig. 4A and B, shows that mAKAP coprecipitates with NCX1 while the other AKAPs tested (79, 95, 121, 149, and 220) did not. The size of mAKAP is 300 kDa and is identified by the specificity of the mAKAP antibody.2 The cardiac lysate probed with the same antibody contained a 300-kDa band as well as the smaller bands (Fig. 4A). Others have also detected the proteolytic fragments of the 300-kDa mAKAP (47).

Localization of NCX1, mAKAP, and PKA-RI in Rat Cardiomyocytes

To identify the NCX1 and associated proteins of the macromolecular complex in situ, we used immunocytochemical techniques to localize these proteins in rat ventricular cardiomyocytes. NCX1 was identified in the sarcolemmal membrane and in the Z-lines of cardiomyocytes (Fig. 5) as already reported (48, 49). Interestingly, mAKAP is also localized in the Z-lines and the dual staining of NCX1 and mAKAP confirmed that both of these proteins are present in the same location in rat cardiomyocytes (Fig. 5A, overlay). The regulatory subunit of PKA, RI has been identified in the Z-lines and also in other regions of the cell (Fig. 5C). The overlay of the RI and NCX1 clearly shows that both of these proteins are colocalized in cardiomyocytes (Fig. 5C). Analysis of the fluorescence intensities in selected regions of the cell indicates that there is clear correspondence between NCX1 and mAKAP (Fig. 5B) and that there is even better support for the matching of PKA-RI and NCX1 (Fig. 5D). The mAKAP and PKA-RI are also expressed in places that do not contain NCX1 and that are also shown in immunofluorescence images.

DISCUSSION

We have presented data demonstrating that the NCX1 protein in cardiac cells is part of a macromolecular complex (Fig. 6). The complex consists of two types of protein kinases (PKA and PKC), two types of phosphatases (PP1 and PP2A), and an anchoring protein (mAKAP). In addition, there is strong evidence that NCX1 also binds to the widely distributed adapter protein, ankyrin (50, 51). The activity of NCX1 has been shown to be regulated by its interaction with a number of ions and molecules (Table I). How this regulation may be organized and controlled by the macromolecular complex is presented below.

Organization of the Macromolecular Complex

The data presented here and by others elsewhere suggest that the intracellular loop is the probable site of phosphorylation by PKA, PKC, and CamKII-dependent phosphorylation. Consequently, the intracellular loop of NCX1 is the most likely region to be associated with mAKAP. Our current thinking is best summarized by the cartoon shown in Fig. 6 that draws on our findings shown in Figs. 1–4 and the findings of others (20, 50, 52–55). In addition to the nine transmembrane regions (TM), there are two pore-forming regions (between TM2 and 3 and between TM7 and 8) and a large intracellular loop between TM5 and 6. TM2, 3, 7, and 8 are organized to form the “transport pore” while the other TM regions are thought to form an...
outer ring (Fig. 6, right panel). There are two short TM linker intracellular regions between TM1 and 2 and between TM3 and 4. The intracellular loops and the C terminus remain as possible sites for anchoring of the macromolecular cluster. We favor the large intracellular loop because it is the site of measured phosphorylation (20) and its clearly established role in NCX1 regulation (33, 34, 56).

mAkAP—The same large 300-kDa AKAP (mAkAP) that is involved in NCX1 signal modulation also targets the cardiac RyR2. RyR2 and mAkAP are linked to each other through leucine/isoleucine-zipper (LZ) regions (57). In analyzing mammalian NCX1 sequences, 7 putative LZ motifs are conserved in sequence and position within the intracellular loop. It remains to be seen whether these motifs are responsible for mAkAP association with NCX1.

pKa—The catalytic subunits and the regulatory subunits were found associated with NCX1. The attachment of the catalytic subunits is facilitated by the regulatory subunits through the AKAP to NCX1. Whether RI binds to NCX1 through mAkAP or through another AKAP is yet to be investigated.

The finding of RI as the regulatory subunit in the NCX1 complex is important. RI subunits are dynamic and developmentally regulated (58). The association of RI with NCX1 complex suggests that the PKA modulation of the NCX1 is more dynamic than previously appreciated and that phosphorylation may be an important signal or regulator during development.

PKC—PKC was found to be associated with NCX1, but we do not know how PKC is attached to the NCX1. The PKC could be linked via mAkAP, but in other systems such as the neuromuscular junction (59), distinct AKAPs link PKA and PKC to their targets. PKCβ in the neuromuscular junction is associated with AKAP250 (also known as gravin) to the postsynaptic membrane. Interestingly, gravin is also reported to be associated with PKA in erythroleukemia cells (60) providing a precedent for the possibility that gravin may bind to both PKA and PKC, but this requires further investigation.

Protein Phosphatases, PP1 and PP2A—We also identified the phosphatases, PP1 and PP2A associated with NCX1. It has been demonstrated in the RyR2 macromolecular complex that these proteins are linked to RyR2 through independent linker proteins. PP1 binds RyR2 through targeting protein, spinophilin, and PP2A binds RyR2 through the targeting protein, PR130. These interactions with RyR2 occur through the LZ regions (57). As noted above, we have identified several LZ regions in the intracellular loop of NCX1 and these regions could be responsible for PP1 and PP2A association with NCX1. Another possibility is that PP1 alone could directly bind NCX1. Others have reported that the PP1 binds directly to ion transporters through specific sequence motifs in the target protein (61–63). We have identified at least four possible PP1 binding regions in the rat NCX1. We doubt the involvement of three of these possibilities based on either their location in the protein or lack of conservation across species. However, there is one region that is conserved by sequence and position in NCX1 of mammalian species and NCX of Drosophila and squid (Fig. 7). This region (asterisk in Fig. 6, left) is an α-helical region that was initially modeled to be a transmembrane segment. More recently, re-examination of the topology now places this region at the end of the intracellular loop (52, 53).

Regulators and NCX1

Table I shows that there are many common features shared by NCX1 and other macromolecular complexes. The theme of macromolecular complexes is increasingly appreciated by recent work investigating how signaling can be targeted locally while many of the critical elements are also seen widely within
the cell. NCX1 itself has important cell-wide (i.e. “global”) influence in overall Ca\(^{2+}\) homeostasis but also has a role in Ca\(^{2+}\) signaling that is more “local” in nature.

**Interaction with Ions**—The macromolecular complex of NCX1 is associated with the intracellular components of NCX1 and probably with the large intracellular loop found between transmembrane (TM) segments 5 and 6. It is this part of NCX1 that has both known binding sites for PP1 and a possible association site with mAKAP (Figs. 6 and 7). The intracellular loop also contains a regulatory Ca\(^{2+}\) binding site, Na\(^+\) association site, sites for H\(^+\) regulation, and a “XIP” binding site. Although definite view on the spatial organization of the intracellular loop will await a structural investigation of this part of the protein, the preliminary data to date suggest that these regions of association in the intracellular loop interact.

**Regulation by Phosphorylation**—Early experiments suggested that ATP played an important regulatory role in NCX1 function (26, 64, 65). Possible roles for ATP were “direct” regulation or through phosphorylation of NCX1. The role of phosphorylation, however, has been uncertain until now. For example, experiments using the giant patch method have found no effect of phosphorylation on NCX1 function (11, 12). In contrast, other studies using both intact cells (20, 66) and biochemical approaches (20, 33) came to the opposite conclusion. Here we provide an explanation of these paradoxical observations. The presence of colocalized kinases and phosphatases make it possible for rapid dephosphorylation through phosphatase activity so that physiological phosphorylation may be absent or not found even when it is present and important in vivo (38, 39). In contrast, if kinases were to dominate the local process, physiological phosphorylation would be apparent.

**Interaction with Kinases**—In this report, we show that the macromolecular complex of NCX1 contains the kinase, PKA holoenzyme consisting of two PKA catalytic subunits and two identical PKA regulatory subunits composed of RI (Figs. 2 and 6). Importantly, only the regulatory subunit RI is found in the complex, whereas both RI and RII are present in the heart cell. Immunofluorescence studies clearly reveal the presence of RI in the Z-lines of rat ventricular cardiomyocytes (Fig. 5). This is the first demonstration of the AKAP targeting of RI in heart.

All previous reports have found RII associated with AKAPs in heart (36, 37, 57).

The macromolecular complex also contains PKC as shown in Fig. 3. Both cloned cardiac NCX1 expressed in CCL39 cells and the neonatal rat cardiac NCX1 showed an increased NCX1 activity when treated with phorbol ester that activates PKC (20, 31). In such experiments, phosphorylation was measured (32). In contrast, studies employing giant patches to measure NCX1 activity did not show any effect of PKC activation (11, 12). The presence of phosphatases in the macromolecular complex may account for these different conclusions.

**Interaction with Phosphatases**—The NCX1 macromolecular complex contains two phosphatases, PP1 and PP2A. The PP1 can be associated with NCX1 through mAKAP or directly to NCX1 (see above). The phosphatase PP2A can be associated with mAKAP through the adapter PR130 (57) or through gravin (59). Similar to PP1, PP2A is also serine-threonine phosphatase that is widely found and tightly regulated. The presence of two phosphatases and two kinases in the NCX1 macromolecular complex provides for both specific and subtle control of function.

**Macromolecular Complexes in Other Systems, Local Control, and Targeting of the NCX1**—Similar to the NCX1 complex described in this report, macromolecular complexes have been reported in heart for t-type Ca\(^{2+}\) channels, RyR2 (the SR calcium release channel), and KCNQ1-KCNK1 K\(^+\) channels (35–37). These complexes and NCX1 complex share the common feature in which the protein kinases and the phosphatases are tied to the central protein. By forcing the colocalization of regulatory enzymes, the central protein can be modulated more quickly and efficiently. The main protein can be organized with respect to other proteins within the cell through association with cytoskeletal proteins. Evidence suggests that NCX1 binds the adapter protein, ankyrin (50, 67), to guide the location of NCX1 within the cell. Ankyrin is thought to interact with spectrin and spectrin with actin. It is not known how ankyrin binds to NCX1. It is also possible that other adapter proteins may also be involved in local positioning of NCX1. It does appear that ankyrin-B is critical to T-tubular targeting of NCX1 (67), and this has important functional consequences.

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**Fig. 6. Organization of the NCX1 macromolecular signaling complex.** Left, the linear arrangement and the current consensus assignment of transmembrane sequence are shown. The asterisk indicates the position of possible PP1 binding site. A top view of the spatial organization is also shown. Right, possible organization of the NCX1 macromolecular complex that includes PKA (PKAc and R1) and PKC as well as mAKAP and phosphatases PP1 and PP2A. The kinases and phosphatases are possibly linked by protein mAKAP. The adapter protein, ankyrin, is shown to link the NCX1 complex with the spectrin-actin cytoskeletal network based on the published work of others.
There is also the possibility that AKAPs can directly bind to the cytoskeleton as was demonstrated for ezrin (68).

Colocalization of NCX1 with mAKAP and PKA-RI—NCX1 has been identified in the sarcolemma and in Z-line/T-tubules by us and others (48, 49, 67). The mAKAP has been localized in the sarcoplasmic reticulum, in the perinuclear region, and in Z-line/T-tubular regions in cells (37, 47, 69). We have identified mAKAP in the Z-lines/T-tubules of adult rat ventricular cardiomyocytes, and mAKAP colocalized with NCX1 in these cells. These findings are significant because the L-type voltage-gated Ca\(^{2+}\) channels are also localized in the T-tubules (70). Similar to the modulation of Ca\(^{2+}\) channels by the β-adrenergic stimulation through PKA activation, NCX1 also has been shown to be influenced (39). The PKA subunit, RI, is found in the Z-lines/T-tubules and is colocalized with NCX1. These immunochemical results support the biochemical findings that NCX1 exists as a macromolecular complex in cardiomyocytes.

**Significance of the NCX1 Macromolecular Complex**

The central role of Ca\(^{2+}\) in EC coupling and cardiac contractility elevates the importance of tight regulation of NCX1, the primary Ca\(^{2+}\) extrusion mechanism in heart. The important local signaling functions carried out by NCX1 also demand careful but flexible and rapid control of its function. The NCX1 macromolecular complex is the first such complex reported for a transporter protein other than a channel. As noted above, virtually all of the proteins with regulatory macromolecular complex involving AKAPs influence intracellular [Ca\(^{2+}\)] like NCX1.

**Acknowledgment**—We thank Cecilia Frederick for help in immunocytochemistry.

**REFERENCES**


**TABLE I**

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**Putative PP1 binding motif**

**Rat NCX1** (774) YVMHFLTVFKVLFVFV

**Rat NCX2** (724) YVMHFLTVFKVLFACL

**Rat NCX3** (710) YVMHFLTVFKVLFACV

**Dros Dmel/Ncx** (752) YVSHFVCLFVKLFAVF

**Squid NCX** (695) YIMHVFCLFKLFAVF

Fig. 7. Conservation of PP1 binding motif in NCX family. Location of the PP1 binding motif (KVXF) in the rat NCX 1, 2, 3, *Drosophila* Dmel/Ncx, and squid NCX. Number in parenthesis corresponds to the first residue in each sequence.

There is also the possibility that AKAPs can directly bind to the cytoskeleton as was demonstrated for ezrin (68).

**Colocalization of NCX1 with mAKAP and PKA-RI—NCX1 has been identified in the sarcolemma and in Z-line/T-tubules by us and others (48, 49, 67). The mAKAP has been localized in the sarcoplasmic reticulum, in the perinuclear region, and in Z-line/T-tubular regions in cells (37, 47, 69). We have identified mAKAP in the Z-lines/T-tubules of adult rat ventricular cardiomyocytes, and mAKAP colocalized with NCX1 in these cells. These findings are significant because the L-type voltage-gated Ca\(^{2+}\) channels are also localized in the T-tubules (70). Similar to the modulation of Ca\(^{2+}\) channels by the β-adrenergic stimulation through PKA activation, NCX1 also has been shown to be influenced (39). The PKA subunit, RI, is found in the Z-lines/T-tubules and is colocalized with NCX1. These immunochemical results support the biochemical findings that NCX1 exists as a macromolecular complex in cardiomyocytes.