**NCX1 phosphorylation dilemma: a little closer to resolution. Focus on “Full-length cardiac Na⁺/Ca²⁺ exchanger 1 protein is not phosphorylated by protein kinase A”**

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**NA⁺/Ca²⁺ EXCHANGER (NCX) proteins constitute a family of solute carriers that provide the major pathway for removal of calcium from the cytosol in exchange for three times as many sodium ions. The vertebrate NCX proteins are expressed as tissue-specific subtypes coded by multiple genes (NCX1, NCX2, and NCX3 in mammals) and further diversified by variable splicing (e.g., NCX1.1, NCX1.2, and NCX1.4). These subtypes show subtle differences in regulation caused by the transported cations and possibly by adrenergic transmitters. β-Adrenergic signaling is mediated by the cAMP-dependent protein kinase A (PKA) or more significantly, CaM kinase II, which in heart muscle can phosphorylate the L-type calcium channels, phospholamban [regulating sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA) activity], the ryanodine receptor, and myofibrils. Because NCX plays such an important role in calcium homeostasis in both the normal and failing heart, possible β-adrenergic-mediated phosphorylation of NCX would be expected to have significant consequences on cardiac contractility. Unfortunately, the issue of adrenergic regulation of NCX remains controversial because of conflicting data in support of direct PKA-mediated phosphorylation of NCX. In this issue of *AJP-Cell Physiol*, using bioinformatics analysis and peptide arrays, Wanicahan et al. (30) present compelling data that mammalian cardiac NCX is not a direct target for PKA.

Early studies using squid axons (5, 6) and sarcolemmal vesicles (2) did not differentiate between direct binding effects of ATP and ATP-dependent phosphorylation. Hilgemann et al. (13, 19, 20) addressed this problem but found no functional changes in NCX current (I_{NCX}) resulting from phosphorylation following application of PKA or PKC catalytic subunits to excised giant patches of cardiac myocytes or in dog cardiac NCX1-expressing *Xenopus* oocytes. While we and others (4) have confirmed the absence of β-adrenergic and ATP effects on cloned mammalian cardiac NCX1 exchanges in various expression systems, some groups have found stimulatory effects in intact mammalian cardiomyocytes (10, 25). Studies using single cells (with well-developed sarcoplasmic reticulum) have been subject to criticism based on the possibility that PKA may exert its effect on NCX indirectly. This stresses the need for simultaneous measurements of I_{NCX} (including its reversal potential), intracellular Ca²⁺ ([Ca²⁺]ᵢ) [carried out at physiological temperatures (25)], and direct measurements of NCX1 phosphorylation. The dual challenge of demonstrating phosphorylation and conducting experiments at physiological temperatures was taken up by Ruknudin et al. (26, 27) who found enhancement of outward I_{NCX} of cardiac (NCX1.1) but not renal (NCX1.3) splice variants (27). Nevertheless, more critical studies by a number of labs showed no stimulation of cardiac NCX1 in rabbit (9) and rodents (18), consistent with our observations in the guinea pig, rat ventricular myocytes, and recombinant dog NCX expressed in human embryonic kidney (HEK)293 cell lines (11).

**Phosphorylation of NCX**

It has been generally challenging to measure directly NCX phosphorylation (4, 8, 31). Two groups (28, 31) have reported that cardiac NCX could be phosphorylated in vitro by PKA, and one group (14) has demonstrated that NCX undergoes in vivo phosphorylation by PKC. The very low number of studies reporting in vivo or even in vitro phosphorylation of mammalian NCX in the last two decades raises the possibility that β-adrenergic stimulation may exert its effect on NCX indirectly (32), if at all.

Some of the most convincing evidence on NCX phosphorylation by PKA comes from a study by Wei et al. (31), where NCX was immunoprecipitated from control and failing hearts and then subjected to PKA-mediated “back phosphorylation.” The level of PKA-mediated in vitro NCX back phosphorylation in failing hearts was found to be twofold lower than NCX from the control heart. The conclusion of this study was that the decreased in vitro back phosphorylation of NCX from failing hearts was the consequence of NCX hyperphosphorylation in the failing myocardium. Unfortunately, the investigators never directly tested this hypothesis by measuring the level of in vitro back-phosphorylated NCX immunoprecipitated from hearts that had been stimulated with isoproterenol. Although there are a number of independent laboratories reporting that β-adrenergic stimulation significantly increases mammalian NCX activity, there are no studies that demonstrate direct in vivo PKA-mediated phosphorylation of NCX in either β-adrenergic-stimulated hearts or isolated cardiac myocytes. Though Ruknudin et al. (27) provide evidence for the upregulation of NCX activity accompanied by increased phosphorylation of the exchanger, once again, what they are speaking of is in vitro NCX back phosphorylation and not a true in vivo assay. Perhaps the reason for not seeing in vivo phosphorylation of NCX emanates from high phosphatase
activity that may dephosphorylate the exchanger during the extraction period, even in the presence of phosphatase inhibitors. If this were the case, it is then puzzling why Wei et al. (31) concluded that NCX isolated from failing myocardium was hyperphosphorylated, whereas NCX isolated from control heart tissue was not, especially when there are studies that show increased PP1 and PP2A phosphatase activity during maladaptive cardiac hypertrophy and failure (3).

Functional Consequences of NCX Regulation in Nonmammalian Hearts

Studies on adrenergic regulation of NCX in nonmammalian hearts have shown unexpected functional twists that provide additional insights into the NCX phosphorylation controversy. Unlike the mammalian heart, NCX serves as both the major Ca\textsuperscript{2+}-influx and Ca\textsuperscript{2+}-efflux pathway, for instance, in frog and shark hearts, where SERCA 2, phospholamban (PLB), or functional Ca\textsuperscript{2+} release pools are either not expressed or absent (22). In the frog ventricular myocytes, isoproterenol was found to strongly suppress I\textsubscript{NCX}, (inhibiting influx of Ca\textsuperscript{2+}) independent of [Ca\textsuperscript{2+}] (7). This finding provides for the well-documented contracture-suppressant effect of the adrenergic hormones on the frog heart, which in the absence of SERCA and its regulation by PKA, had been somewhat puzzling to account for (17, 21, 22). The β-adrenergic agonist’s simultaneous enhancement of calcium current (I\textsubscript{Ca}) and suppression of I\textsubscript{NCX} may enable the frog ventricle to develop force rapidly at the onset of depolarization (enhancement of I\textsubscript{Ca}) and to relax the contraction faster later in the action potential (suppression of I\textsubscript{NCX}). This unique, adrenergically induced temporal shift in Ca\textsuperscript{2+} influx pathways during the action potentials may have evolved in response to virtual absence of SERCA-PLB complex and absence of significant intracellular Ca\textsuperscript{2+} release pools in the frog heart. Interestingly, cloning the frog cardiac NCX revealed an insertion of 9 amino acids with a Walker A, ATP-binding motif, or P-loop [Exon X (15)]. The recombinant frog NCX showed voltage-dependence, divalent (Ni\textsuperscript{2+}, Cd\textsuperscript{2+}) inhibition and sensitivity to cAMP (11, 29) characteristic of native exchanger in frog myocytes (7). Expression of recombinant dog heart NCX1 or a frog mutant NCX lacking the 9 amino acid exon showed no cAMP-dependent regulation of Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} uptake or suppression of I\textsubscript{NCX}, suggesting that the presence of the P-loop in the NCX was critical for the observed cAMP-dependent functional differences between the frog and mammalian hearts (29). These authors also reported that, when the putative PKA site was disrupted on the P-loop either by deletion or by replacing Ser\textsubscript{374} with a Gly, the cAMP-mediated suppression of I\textsubscript{NCX} was somewhat smaller and more transient than what was observed in the wild-type frog NCX (11, 29). Nevertheless, this study also failed to show experiments demonstrating direct phosphorylation of the frog NCX at Ser\textsubscript{374}. It should be noted that the differences in I\textsubscript{NCX} seen in the deletion and replacement of Ser\textsubscript{374} with Gly do not unequivocally prove that Ser\textsubscript{374} is phosphorylated but could reflect subtle changes in the secondary structure of NCX.

Bioinformatics and Peptide Substitution

Wanichawan et al. (30) rigorously take us one step closer to settle the controversial question of whether NCX undergoes phosphorylation by PKA. Using bioinformatics analysis and peptide arrays, they screened the NCX sequence for putative PKA phosphorylation sites. Several of the peptides containing these sites can be phosphorylated by PKA in vitro, but mutational analysis demonstrated that only one PKA site Thr\textsubscript{731} was actually the target. The intriguing observation, however, was that this novel Thr\textsubscript{731} phosphorylation site (IKKT) was not accessible in intact full-length NCX. Most importantly, even though some of the NCX peptides could be phosphorylated, the immunoprecipitated full-length NCX-GFP or even the calpain- or caspase-3-digested NCX was not phosphorylated by PKA in vitro. This conclusion of the authors is consistent with the most rigorous functional studies in single mammalian cardiomyocytes where isoproterenol, forskolin, or cAMP analogs were reported to fail to alter I\textsubscript{NCX} significantly.

This nevertheless begs the question as to the reason why some labs find functional changes in the NCX current on β-adrenergic stimulation. Perhaps the simplest explanation to this conundrum is the difficulty of measuring NCX function through electrophysiological experiments. Choosing the “ideal” conditions in patch-clamped myocyte to measure a small current that may easily be contaminated by fluxes of other ions, such as: 1) monovalents traversing through the L-type calcium channels, 2) Cl\textsuperscript{-} currents through cystic fibrosis transmembrane conductance regulator channels, or 3) currents through membrane leak and TGF-β receptor interacting protein channels, or 4) transient intracellular Ca\textsuperscript{2+} changes complicate the interpretation. In addition, intracellular buffering of calcium remains an enigma, where exogenous powerful buffers, some highly pH dependent, are included in cell-dialyzing patch pipette solutions. Although this may serve as a reasonable explanation in mammalian myocardium, the functional studies on frog and shark ventricular myocytes are more compelling, because they not only show strong and reversible effects by isoproterenol, forskolin, and cAMP on significantly larger NCX currents generated in native frog and shark myocytes or recombinant clone of frog- and shark-NCX expressed in Xenopus oocytes or HEK293 cell lines, but also because they serve as a mechanistic explanation for well-documented effects of isoproterenol in intact frog ventricular strips (17, 22). Despite these compelling functional findings, the cloned frog-NCX also failed to show any indication of phosphorylation. Putting the two sets of data together it is intriguing to consider that phosphorylation of an intermediary protein may be mediating the effects of isoproterenol in the frog heart. Perhaps the presence of exon X in the frog NCX provides a binding site for such an intermediary protein, and its absence in the mammalian hearts does not allow for docking of such an intermediary protein.

Predictions and Future Possibilities

It is tempting to speculate how the new results of Wanichawan et al. (30) may be interpreted from the current understanding of the functional organization of the NCX molecule as shown schematically in Fig. 1. In this schematic
NCX1 PHOSPHORYLATION DILEMMA

Where Do We Go From Here?

What domains mediate β-adrenergic responsiveness of NCX in nonmammalian species? Perhaps comparative analysis of nearly hundreds of NCX sequences representing numerous phyla may help identify which domains are required for β-adrenergic responsiveness, and whether the expression of such domains correspond closely with evolutionary development of adrenergic regulation in the heart. This approach could be expanded to include sequence comparison of the closely related NCX1 exchangers. Sophisticated modeling approaches, especially when combined with structural information gleaned from evolving crystal structure of the intracellular regulatory loop of NCX, could provide clues as to the molecular requirement for possible mammalian adrenergic regulation/phosphorylation. Where adrenergic regulation of NCX is well established, construction of shark/mammalian NCX chimeras, examining the consequences of β-adrenergic stimulation in a transgenic mouse with shark NCX1, or even more elegantly, a knockin of the NCX1 β-adrenergic responsive domain, might provide insights into the interplay of adrenergic regulation of NCX vis-à-vis the cellular calcium homeostasis and cardiac contractility. Perhaps another productive line of investigation would be to pursue the identification of an intermediary factor or protein that maybe the conduit for the β-adrenergic responsiveness of NCX. One can utilize coimmunoprecipitation in the shark NCX1 transgenic mouse model or fluorescence resonance energy transfer to determine whether PKA directly interacts with NCX1 following β-adrenergic stimulation and possibly identify other factors that complex with NCX using mass spectroscopy. As new imaging and single molecule technologies evolve it is likely that, in the not too distant future, one will be able to follow the unstructured loop of β₁ [which has also another additional S₂ at the poorly conserved loop within α_CAT (16)]. Interestingly, the critical residues found by Wanichawan et al. (30) are located between β₂ and α_CAT, where in vitro phosphorylation of Thr₇₃₁, dependent on Ser₇₂₂ and Thr₇₂₃ in synthetic peptides, was found to occur but was not available in fully intact mammalian cardiac NCX. Considering these observations we suggest that PKA-mediated regulation of NCX occurs only when a number of unstructured linker sequences are of adequate length to allow the more rigid β₁, β₂, and α_CAT structures to assume an open configuration where critical serine (Ser₃₈₈ and Ser₇₂₂) and threonine residues (Thr₇₂₃ and Thr₇₃₁) are exposed to allow interactions with PKA and its cofactors. This unifying hypothesis would gain strength if: 1) nuclear magnetic resonance and X-ray diffraction analysis confirm an α-catenin-like structure for the suggested fragments of NCX; 2) the isoforms of cardiac NCX (frog, shark) that generate cAMP-dependent currents and Ca²⁺ fluxes could be phosphorylated at Thr₇₃₁, and 3) Ser₃₈₈, which appears to be located close to Thr₇₃₁, is shown to affect the Thr₇₃₁ phosphorylation. When interpreted in this way, it is possible that the in vitro phosphorylation of Thr₇₃₁ (30) is not just another false lead in the quest to understand PKA-mediated regulation of cardiac NCX but a crucial piece in a puzzle that presently seems to contain many incongruous observations.

The coupled exchange of calcium and sodium ions directly involves approximately nine membrane-spanning α-helices (top) that is regulated by a long cytoplasmic loop with highly conserved regions. Principal among these are two homologous seven-stranded β-sandwich structures [β₁ and β₂ (1, 12, 23)] with Ca²⁺ binding sites at one end (Ca²⁺) and unstructured loops extending at the other. As suggested by Hilge et al. (12), it is possible that an α-catenin-like domain (α_CAT) with four anti-parallel α-helices mediates signal transduction from the Ca²⁺-sensing to the membrane-spanning domains. Elaborating on this idea, in Fig. 1 we suggest that the amino acids that may form the fourth α-helix of the hypothetical α_CAT structure could be found downstream from β₂ (rather than be partially missing just before β₁). This new rearrangement provides additional sequence homology between NCX and α-catenin, places the resulting α_CAT domain more firmly as a linker between the Ca²⁺-binding and membrane-spanning domains, and may serve to explain regulation of NCX by phosphorylation in terms of different molecular determinants (shown in green in Fig. 1). In this scheme, PKA-mediated regulation of frog cardiac NCX depends on exon X, which further adds to the variably spliced region (exons A/B-CDEXF) of β₂. Thus the location of Ser₃₈₈ (between α_CAT and β₁; corresponding to Ser₇₇₄ of frog cardiac NCX) becomes available for regulation. Similarly, PKA-mediated bimodal regulation of shark cardiac NCX depends on an addition of a S₁ region to the...
movement of PKA as it docks on NCX protein or an associated protein in intact myocytes, providing direct evidence on the regulation of this fascinating and ancient gene.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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