INSULIN EFFECTS ON CARDIAC NA⁺/CA²⁺ EXCHANGER ACTIVITY: ROLE OF THE CYTOPLASMIC REGULATORY LOOP

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Running head: Insulin regulation of Na⁺/Ca²⁺ exchange

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Insulin can alter myocardial contractility, in part, through an effect on the cardiac sarcolemmal Na⁺/Ca²⁺ exchanger (NCX), but little is known about its mechanism of action. The large cytoplasmic domain (f-loop) of NCX is required for regulation by various intracellular factors and we have previously shown that residues 562-679 are determinants of NCX inhibition by exchanger inhibitory peptide (XIP). Here we show that the same f-loop deletion eliminates the enhancement of NCX current by insulin and we examine the signal pathways involved in the insulin response. NCX current ($I_{NCX}$) was measured in freshly isolated or cultured (up to 48 hrs) adult guinea-pig myocytes, and in myocytes expressing canine NCX1.1 with the 562-679 f-loop deletion (NCXΔ562-679) via adenoviral gene transfer. $I_{NCX}$ was recorded by whole-cell patch clamp as the Ni²⁺-sensitive current at 37°C with intracellular Ca²⁺ buffered. Insulin (1µM) increased $I_{NCX}$ (at +80mV) by 110% and 83%, in fresh and cultured myocytes, respectively, while in myocytes expressing NCXΔ562-679, the response was eliminated (with 100µM XIP included to suppress any native guinea-pig $I_{NCX}$). The insulin effect on $I_{NCX}$ was not inhibited by wortmannin, a nitric oxide synthase inhibitor, or disruption of caveolae, but was blocked by chelerythrine, implicating protein kinase C, but not phosphatidylinositol-3-kinase, in the mechanism. The insulin effect was also not additive with PIP₂-induced activation of NCX. The finding that the 562-670 f-loop domain is implicated in both XIP and receptor-mediated modulation of NCX highlights its important role in acute physiological or pathophysiological regulation of Ca²⁺ balance in the heart.

Insulin is an essential hormone for the control of blood glucose concentration. In the heart, insulin affects many physiological and pathological functions, including energy metabolism, contractility (1,2), ion transport (3), cardiac protection (4), hypertrophy (5) and cardiomyopathy in diabetes (6). The sarcolemmal Na⁺/Ca²⁺ exchanger (NCX) has been shown to be an insulin-sensitive target - NCX protein and mRNA expression levels are significantly depressed in some diabetic animal models (7,8) and NCX activity, but not mRNA, was decreased in streptozotocin-treated neonatal rats(9). NCX regulation by insulin has also been proposed as one of the underlying mechanisms responsible for the positive inotropic effect of insulin on the failing myocardium(10,11). Because NCX is the main mechanism for removing Ca²⁺ from the cell, accounting for up to 30% of the total systolic Ca²⁺ removal in large animals, a significant reduction in exchanger activity could increase intracellular Ca²⁺ and may contribute to diabetic cardiomyopathy, as a result of altered diastolic Ca²⁺ removal (12). The pleiotropic actions of insulin are mediated by a cascade of parallel signaling pathways within the cell. The binding of insulin to its receptor results in the activation of its intrinsic protein tyrosine kinase activity with several downstream effects. One of the earliest steps of insulin signaling involves phosphatidylinositol-4,5-bisphosphate (PIP₂). This membrane associated lipid is hydrolyzed by phospholipase Cγ (PLCγ) to generate two second messengers, diacylglycerol (DAG), which activates protein kinase C, but not phosphatidylinositol-3-kinase, in the mechanism. The insulin effect was also not additive with PIP₂-induced activation of $I_{NCX}$. The finding that the 562-670 f-loop domain is implicated in both XIP and receptor-mediated modulation of NCX highlights its important role in acute physiological or pathophysiological regulation of Ca²⁺ balance in the heart.
domain kinases, and the small RAS GTPases to activate gene transcription responses.

The involvement of PIP₂ in the insulin response is of particular interest with respect to potential regulation of NCX, since PIP₂ has already been shown to modulate the activity of NCX in giant patch experiments (15) and influence NCX trafficking to the membrane(16). Hydrophobicity analysis of the NCX primary sequence suggests 9 transmembrane regions with a large hydrophilic region between segments 5 and 6 (f-loop) (17). Ion exchange and transport functions are associated with the transmembrane segments whereas exchanger regulation appears to be mediated by the large (520 amino acids) cytoplasmic domain, f-loop. This intracellular loop is essential for regulation of NCX activity by a number of intracellular factors (see reviews (18,19)). At the N-terminal end of the f-loop, there is a domain referred to as the endogenous exchanger inhibitory peptide (XIP) region (20). When a synthetic peptide with the same sequence as the endogenous XIP region is applied to the intracellular side of the membrane, NCX function is potently inhibited (20). PIP₂ activation of the exchanger is also thought to involve the XIP domain of NCX(21).

Previous evidence from our laboratory indicated that f-loop residues 562-679 contain sites involved in the regulation of the NCX by the endogenous XIP region (22). Deletion of these amino acids eliminated XIP-induced inhibition of NCX in either non-cardiac cell lines or in adult guinea-pig cardiomyocytes (22). Therefore, the aims of present study were to evaluate the effects of insulin on the native NCX current (I NCX) and to examine the role of the 562-679 f-loop deletion on the insulin response in intact adult myocytes.

**Experimental Procedures**

**Viral vectors**

The coding sequence for the canine wild-type NCX (NCX1.1) was a gift of Dr. Kenneth Philipson (University of California, Los Angeles). The deletion mutant Δ562-679 was constructed, as previously described (22), by deleting amino acids 562-679, corresponding to the sequence FQNDEI...to..EHTKLEV of NCX using an Exsite polymerase chain reaction-based site directed mutagenesis kit from Stratagene (La Jolla, CA).

**Adult cardiac guinea pig myocyte experiments**

Animal protocols used were in accordance with the Guide for Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and were approved by the Johns Hopkins Animal Care and Use Committee.

Isolated guinea pig ventricular myocytes were obtained by enzymatic digestion as previously described (23). Freshly isolated myocytes were cultured in Dulbecco’s Modification of Eagle’s Medium (DMEM; Mediatech, Herndon, VA) supplemented with 5% fetal bovine serum (FBS, Invitrogen, San Diego, CA), 1% penicillin-streptomycin (Invitrogen), and 15 mmol/L HEPES (Life Technologies, Inc., Gaithersburg, MD), pH 7.4, on laminin-coated cover slips in a 5% CO₂ incubator at 37°C. After 2 hours, the myocytes were either used for patch-clamp experiments or infected with adenovirus carrying the deletion mutant gene of NCX (pAdEcd-Δ562-679), together with a bicistronic vector containing a cytomegalovirus (CMV)-driven green fluorescent protein (GFP) sequence and a Drosophila/Bombyx driven ecdysone receptor (pAdCGI-DBEcR) at a ratio of 10:1 as described previously (24). The fluorescence emission of the GFP reporter enabled us to identify transfected cells. Viruses were added to myocytes 2 hours after being plated on coverslips. After 1 hour, the medium was changed for DMEM supplemented with 10% FBS and 1% penicillin/streptomycin with the addition of ponasterone A (1-5 µg/ml; Invitrogen) in order to induce the expression. Medium with ponasterone A was exchanged every 24 hours. Experiments were carried out 36-50 hours post-induction.

**Whole-Cell Patch-Clamp recording**

For I NCX measurements, cover slips were mounted in a heated recording chamber (37°C) on the stage of a fluorescence microscope (Nikon Eclipse TE300) and superfused with external solution contained (in mmol/L): NaCl 128, CsCl 10, CaCl₂ 2, MgCl₂ 1, Na-HEPES 10, Glucose 10, pH=7.4 with CsOH. The solution was K⁺-free to block inward rectifier K⁺- and Na⁺-K⁺-ATPase currents and also contained (in µmol/L): nifedipine
10, 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulfonic acid (SITS) 100 and ouabain 0.5 in order to block L-type Ca\textsuperscript{2+}, Cl\textsuperscript{-} and Na\textsuperscript{+}-K\textsuperscript{+}-ATPase currents, respectively.

The internal solution contained (in mmol/L): CsCl 120, CaCl\textsubscript{2} 3, MgCl\textsubscript{2} 0.5, Na-HEPES 20, Mg-ATP 5, K-BAPTA 5, pH=7.25. The free internal calcium concentration ([Ca\textsuperscript{2+}]) was 200 nmol/L calculated using Maxchelator program (25). The pipette-to-bath liquid junction potential was –2.7 mV and was corrected. Where indicated, XIP (RRLLFYKYYKRYRAGKQRG) 100 µmol/L, synthesized and purified by the protein synthesis core facility of Johns Hopkins University, was added to the pipette solution. In some experiments phosphatidylinositol-4,5-biphosphate (PIP\textsubscript{2}) was added (30 or 60 µmol/L) to the pipette solution. This solution was sonicated before use for 15 minutes. For cholesterol depletion to disrupt the caveolae(26), the myocytes were treated with 1 mM methyl-β-cyclodextrin (MβCD) in DMEM supplemented with 5% FBS and 1% penicillin/streptomycin for 30 minutes at 37°C.

Cells were voltage-clamped at a holding potential (E\textsubscript{H}) of –40 mV, with families of pulses applied from +80 to –80 mV (in 20 mV steps) for 300 ms at 0.5 Hz. After each pulse, voltage returned to E\textsubscript{H}. The NCX blocker NiCl\textsubscript{2} (Ni\textsuperscript{2+}, 10 mmol/L) was added to the superfusing solution at the end of each experiment to obtain the Ni\textsuperscript{2+}-sensitive current, hereafter referred to as I\textsubscript{NCX} in all figures, was increased more than two-fold across the entire voltage range by insulin, as shown in the current density vs. voltage (I-V) plots (Figure 1E). A significant increase was observed for both reverse-mode (at +80mV +128.4±28.6%, n=9, p<0.05) and forward mode I\textsubscript{NCX} (at –80mV +147.4±38.7%, n=9, p<0.05) by 10 minutes after the insulin addition. Control currents recorded over the same time period in the absence of insulin did not show any significant change in I\textsubscript{NCX} (data not shown).

The average reversal potential, estimated as the zero current potential of the Ni\textsuperscript{2+}-sensitive current (E\textsubscript{rev}) of NCX, was –40.7±4.7 mV and –34.9±2.6 mV (n=9, NS), in the absence and presence of insulin, respectively.

Previous evidence indicates that, in intact adult cardiomyocytes, f-loop residues 562-679 contain the regulatory sites for the endogenous exchanger inhibitory peptide (XIP), but not those involved in Ca\textsuperscript{2+} activation of NCX (22). In order to examine if that portion of the exchanger participated in the insulin effect, in vitro adenoviral gene transfer of canine NCX1.1 with the amino acids 562-679 of f-loop deleted (NCX\textsubscript{Δ562-679}) to adult guinea pig myocytes was performed. The cumulative I-V plots of I\textsubscript{NCX} for non-infected cultured myocytes (Figure 2A), myocytes expressing NCX\textsubscript{Δ562-679} (Figure 2B), and myocytes expressing NCX\textsubscript{Δ562-679} with XIP in the pipette solution (Figure 2C) were determined in

All data are presented as means±SEM. Statistical analysis between groups was performed by a paired or unpaired Student’s t-test. For multiple comparisons, ANOVA analysis followed by the Newman-Keuls post hoc test was used. A value of p<0.05 was taken to indicate statistical significance.

Results

The insulin response and the role of the f-loop

Membrane currents were evoked by depolarizing pulses between +80mV and -80mV from a holding potential of -40 mV in guinea-pig myocytes at 37°C at baseline (control, Figure 1A), in the presence of insulin (1 µmol/L, Figure 1B), and after the addition of NiCl\textsubscript{2} (10 mmol/L, Figure 1C). The mean Ni\textsuperscript{2+}-sensitive current, hereafter referred to as I\textsubscript{NCX} in all figures, was increased more than two-fold across the entire voltage range by insulin, as shown in the current density vs. voltage (I-V) plots (Figure 1E).

Statistics
the presence or absence of insulin. XIP was included in order to block any remaining native guinea pig I\_INCX, which was predominantly replaced by the mutant NCX more than 24 hours after viral transduction.

Basal I\_NCX density in all groups and at all voltages tested did not differ significantly when fresh myocytes, non-infected cultured myocytes, myocytes expressing N\_INCX\_562-679, myocytes expressing N\_INCX\_562-679 with XIP in the pipette solution and myocytes expressing wild type canine NCX1.1 were compared (Figure 2D; I\_INCX at +80mV: 2.33±0.57 pA/pF, 3.81±0.95 pA/pF, 2.78±0.65 pA/pF, 4.42±0.53 pA/pF, 3.14±0.68 pA/pF, respectively, NS). The insulin effect in both NCX modes was still present in non-infected cultured myocytes (increase at +80mV +65.22±17.67%; at -80mV +172.5±59.9%, p<0.05, n=6) and in cultured myocytes expressing wild type NCX1.1 (increase at +80 mV +54.48±15.14, at -80 mV +65.2±19.9%, p<0.05, n=9; N.S.). The insulin effect on the NCX was abolished with XIP (increase at +80mV +32.2±14.5% and at -80mV, +31.2±19.9%; n=8; N.S. vs. control). This residual insulin response was largely eliminated in the presence of XIP (increase at +80mV: +17.0±10.5%; increase at -80mV: +21.5±17.5%; n=9; N.S.). The NCX conductance (G\_INCX), calculated as the linear slope of the I-V plots, increased significantly with acute insulin treatment in fresh or non-infected cultured myocytes but not in myocytes expressing NCX\_562-679 (Figure 2D). The findings indicate that amino acids 562-679 are required for the insulin effect. As shown in figure 2D, the insulin effect is still present in wild-type canine NCX1.1 expressing myocytes, indicating that the viral transduction itself did not alter the insulin effect. The current in non-infected cultured myocytes was similar to the current in wild-type NCX1.1-expressing myocytes (at +80 mV ≈3 pA/pF), indicating that the total NCX activity remains constant after the native protein is replaced by viral transduction.

Insulin signaling pathways
Our next objective was to determine which of the multiplicity of intracellular signaling pathways activated by insulin might be involved in the effect on the I\_NCX. Earlier reports have suggested that a fraction of the NCX may reside in caveolae(27), in which compartment also resides the components of the nitric oxide synthase pathway. Insulin has been shown to increase NO production in vascular endothelial cells(11), through a mechanism involving phosphorylation of the synthase by Akt (27,28), which is activated by the PI3-kinase pathway. Therefore, we first investigated whether the insulin effect on I\_NCX in adult cardiomyocytes was altered when the nitric oxide synthase (NOS) was inhibited with L-NAME (5 mmol/L). L-NAME treatment had no effect on basal I\_NCX and it did not prevent the enhancement of NCX conductance by insulin (Figure 3). Furthermore, although cholesterol chelation by Methyl-β-cyclo dextrin (MβCD) has been used previously to disrupt lipid rafts and caveolae to prevent ion channel recruitment to the membrane(26), and to disrupt the caveolin-3/NCX protein-protein interaction(16,29), this agent did not significantly affect basal or insulin-stimulated I\_NCX (Figure 4).

We further investigated whether the NCX response was mediated by insulin activation of PI3-kinase by pretreating myocytes with the inhibitor wortmannin (WM). WM significantly enhanced G\_NCX in the absence of insulin, but the insulin effect on NCX was still present. The maximal G\_NCX with Ins and WM+Ins were not different (3.2±0.3 and 3.8±0.6 nS, respectively, NS), indicating that the PI3-kinase pathway did not mediate the insulin effect on the NCX (Figure 5).

Insulin activates phospholipase C to initiate PIP\_2 hydrolysis, which leads to the activation of PKC. Pretreatment of myocytes with the PKC inhibitor chelerythrine (Che; 2.5 µmol/L) blocked the insulin-mediated increase in G\_NCX (Figure 5B).

Because PIP\_2 is a known modulator of NCX activity and is also implicated in both the PI3-K and PLC pathways, we next examined the effects of manipulating the intracellular PIP\_2 levels on basal and insulin-stimulated I\_NCX. Cells dialyzed with PIP\_2 (30 or 60 µmol/L) had larger basal NCX current in the absence of insulin (Figure 6) confirming that PIP\_2 is an important modulator of the I\_NCX in adult cardiomyocytes. While 10 µmol/L PIP\_2 (data not shown) was insufficient to increase I\_NCX, 30 and 60 µmol/L PIP\_2 increased G\_NCX (Figure 6C) to similar levels. Moreover, the insulin effect was small with 30 µmol/L PIP\_2 and was not significant with 60 µmol/L PIP\_2 (Figure 6A-C), that is, the stimulatory effect of insulin was not additive to that of PIP\_2.
The effects of insulin on $G_{NCX}$, expressed as a % increase of basal values, highlights the effects of the different experimental protocols used in this study (Figure 7).

Discussion

In the present study, we demonstrate that insulin can increase $I_{NCX}$ in native adult ventricular myocytes and in cultured myocytes. Deletion of amino acids 562-679 in the cytoplasmic f-loop of NCX eliminated the insulin response. The insulin effect was not mediated by PI3-kinase activation, nor did it involve NOS activation or recruitment of NCX from caveolae. The insulin-mediated increase in NCX conductance was prevented by inhibition of protein kinase C and was not additive with the stimulatory effect of PIP2 on NCX.

NCX in diabetes and the effect of insulin

A decrease in cardiac NCX activity in diabetic animals has been reported by a number of groups (7,8) and has been suggested as a mechanism contributing to diastolic dysfunction and cardiomyopathy in diabetes. In some models, both mRNA and protein levels of NCX are decreased (7); however, the role of acute modulation of NCX by insulin and/or high glucose without an effect on NCX mRNA has been suggested to underlie the altered activity in other models (9,30). Direct measurement of the acute effect of insulin on $I_{NCX}$ under controlled patch-clamp conditions has not been previously reported, but earlier studies have shown that insulin, as well as other agents that induce PI hydrolysis (31), can increase NCX activity within minutes, measured as the Na+ dependent component of 45Ca2+ uptake by sarclemmal vesicles (32-34). Acute enhancement of NCX activity by insulin (34), platelet-derived growth factor-(PDGF-BB), or insulin-like growth factor (IGF-1)(35), can be suppressed by PKC inhibitors. These findings are consistent with the present results showing that PKC inhibition with chelerythrine eliminated the insulin effect on $I_{NCX}$.

Enhanced NCX activity has also been indirectly implicated in the positive inotropic effects of insulin on the failing human myocardium (10,36). Hsu et al (10) reported that insulin increased isometric twitch force of explanted muscle strips from failing human hearts by ~45%. The effect could be partially prevented by blockade of SR Ca2+-ATPase, inhibition of Na+-H+ exchange, or inhibition of Na+-Ca2+ exchange, although L-type Ca2+ currents in isolated myocytes were also shown to be increased by insulin. Similarly, Von Lewinski et al (36) observed an increase in contractility in failing human myocardium in response to insulin, and they concluded that increased reverse-mode NCX-mediated Ca2+ uptake was a key factor in the response. In contrast to the present findings, these authors found that wortmannin inhibited the response and PKC inhibition did not. There could be several possible explanations for the differences between the latter study and our findings, the most obvious being that the latter study was carried out in intact failing muscle, in which many alterations in Ca2+ handling and signaling are known to occur. Moreover, $I_{NCX}$ was selectively examined in the present work, while previous investigations involved the more global output of contractile force, involving the integration of effects on many interacting Ca2+ fluxes. Also, our findings show that, due to the significant effect of wortmannin on the basal current (which may be due to an effect on basal membrane PIP2 turnover), the insulin effect on $I_{NCX}$ only appears to be diminished in the presence of wortmannin when expressed as a percent increase from baseline (only percent changes are reported in the previous study). The maximal insulin-stimulated $G_{NCX}$ was identical to that in the absence of wortmannin. Taken together with the lack of effect of other downstream effectors of PI3-kinase on the insulin response, we conclude that PI3-kinase activation is not involved.

$PIP_2$ levels and $I_{NCX}$

Local PIP2 is proposed to regulate ion transporters and channels by electrostatic modulation, either directly by binding to intracellular components of the transport protein or indirectly by altering interactions between the protein and other regulatory units (37). When the cells were dialyzed with different PIP2 concentrations (30 or 60 µmol/L), we showed that the basal current was larger when compared to controls, indicating that the PIP2 level is an important modulator of the $I_{NCX}$. Hilgemann et al (15) reported that the level of membrane PIP2 exerts a strong influence on Na-dependent inactivation of NCX in giant cardiac membranes patches, i.e., an
increase in membrane PIP2 levels stimulates exchanger activity by eliminating inactivation. Additionally, He et al (38) reported an interaction between the cationic endogenous XIP region and PIP2 in giant excised patches from *Xenopus oocytes*, and, when that interaction is present, the activity of the exchanger is higher because the Na-dependent inactivation is reduced. Shannon et al (24) described a model where the membrane environment and XIP domain may control NCX activity. Their model proposed that in the presence of negatively charged phospholipids the XIP domain (positively charged) associates with the membrane. This prevents interaction with the XIP binding site on the protein. In the absence of negatively charged phospholipids, the XIP domain is free to interact with the binding site and thus decrease NCX activity. In this regard, our previous results have shown that amino acids 562-679 are required to observe inhibition of NCX current by exogenous XIP in intact cardiomyocytes (22). This could be explained by a model in which the endogenous XIP domain, upon displacement from membrane PIP2 might interact with the distal f-loop 562-679 region. This interaction would be absent in NCXΔ562-679. Similarly, disruption of the PIP2/XIP/562-679 axis could potentially explain why the insulin effect was absent in NCXΔ562-679. Further investigation will be necessary to elucidate the precise molecular mechanism of the response, but the observation that high concentrations of PIP2 increased \( G_{\text{NCX}} \) to a level similar to the maximum insulin-stimulated conductance, and insulin could not increase the current more, suggests a mechanism involving modulation of PIP2 in the membrane by insulin. However, the paradox of why a receptor linked to PIP2 hydrolysis might end up stimulating a PIP2-activated transporter needs to be resolved. One potential explanation could involve a negative feedback effect of protein kinase C activation on phospholipase C (see below).

**Protein kinase C**

The insulin effect on NCX was almost completely eliminated with chelerythrine pretreatment, implicating the PKC pathway. This effect is consistent with previous studies showing that direct activation of PKC (e.g. with phorbol ester or phorbol dibutyrate), or activation of G-protein linked receptors coupled to PI hydrolysis, upregulates NCX (see (19) for review). NCX contains serines that are phosphorylated *in vivo* in a PKC-dependent manner, but Iwamoto et al (39) reported that PKC-dependent regulation of NCX activity does not require direct phosphorylation of NCX. However, deletion of almost the entire central cytoplasmic loop (246-672) or mutation of the endogenous XIP domain can eliminate PKC-dependent modulation of NCX (40). Taken together with the present findings, an important interaction between the proximal (XIP) and distal (562-679) portions of the f-loop is indicated. Recent studies of chimeras of alternatively spliced (A and B) regions of NCX1 with NCX2 also support an interaction of the XIP and distal f-loop domain that affects Na+-dependent inactivation of NCX(41) - the region of alternative splicing is included in the 562-679 deletion. One possibility that has been previously mentioned (19) to explain why PKC modulates NCX, but the PKC sites on the exchanger are not required, would be that an ancillary target of PKC interacts with the f-loop of NCX to bring about the effect. Another possibility could be that PIP2 levels might be influenced by PKC activation. Although the initial effect of receptor activation would be to break down PIP2, it is possible that PKC activation could mediate a negative feedback effect on PI hydrolysis over several minutes of stimulation to bring about the positive effect on \( G_{\text{NCX}} \). Inhibition of G-protein-coupled phosphoinositide breakdown by PKC activation has been previously reported (42,43).

**Replacement of native NCX with adenoviral NCX constructs**

In the present study, adenoviral gene transfer of the wild-type canine NCX1.1 or the 562-679 deletion mutant of NCX was performed in guinea pig cardiac myocytes that already express native NCX. An important consideration is how much of \( I_{\text{NCX}} \) is related to native and adenovirally-expressed NCX, respectively, and whether adenoviral expression itself alters the insulin response. In myocytes expressing the Δ562–679 mutant, \( I_{\text{NCX}} \) was almost completely insensitive to XIP, consistent with our previous report (22), indicating that native NCX is largely replaced after 24-48 hours of expression. Similarly, using the same expression method, mutants that shifted the Ca2+ sensitivity of \( I_{\text{NCX}} \) (e.g., Δ498-510) showed a single population with a characteristic \( K_m \) for Ca2+ (22). Moreover, a
robust insulin response was obtained in myocytes expressing the wild-type canine NCX1.1, verifying that adenoviral expression does not alter the insulin signaling pathway.

With respect to the elimination of exogenous XIP sensitivity observed in intact myocytes, it should be noted that preliminary evidence indicates that the NCX 562–679 deletion mutant remains sensitive to exogenous XIP when excised giant patches are studied after its expression in oocytes (L. Hryshko, personal communication), and XIP sensitivity was also observed when most of this region was eliminated in giant patch experiments using split exchanger constructs (44). These differences suggest that NCX regulation may be altered depending on how the current is measured, perhaps due to changes in PIP$_2$-protein interactions in the excised patch (15). Additional investigation will be required to resolve this issue.

**Concluding remarks**

While several mechanistic questions remain to be answered regarding the details of the NCX f-loop interactions leading to the activation of NCX current in response to insulin, the results strongly implicate protein kinase C, and not PI3-kinase in the response. An advantage of the present study is that the NCX current was strictly isolated from other confounding membrane currents or Ca$^{2+}$ handling processes. In addition, intracellular Ca$^{2+}$ was heavily buffered with 5mM BAPTA, so it was unlikely that the effect was due to an indirect insulin-mediated change in cytoplasmic Ca$^{2+}$ leading to activation of the exchanger or an alteration of intracellular Na$^+$. The specificity of the response was reinforced by deletion of the NCX distal f-loop, spanning a region known to disrupt XIP inhibition of the current. The findings suggest that insulin-dependent regulation of NCX is an important physiological modulator of cellular Ca$^{2+}$ homeostasis. Since NCX is downregulated in diabetes and insulin resistance has also recently been correlated with the transition from cardiac hypertrophy to failure(45), this mechanism might have significant pathophysiological consequences as well.
REFERENCES

FIGURE LEGENDS

Figure 1: A-C) Families of representative whole-cell currents evoked by depolarizing pulses between +80 to -80 mV from a holding potential of -40 mV recorded in a myocyte 10 minutes after establishing the whole cell configuration (control; panel A), and after the successive additions of insulin (Ins, 1 \( \mu \)mol/L; panel B) and NiCl\(_2\) (Ni\(^{2+}\), 10mmol/L; panel C). Pulses (300 ms in duration) were delivered at 0.5 Hz. D) Voltage dependence of the average current density measured in 9 myocytes exposed to insulin (1 \( \mu \)mol/L) and Ni\(^{2+}\). E) Average current-voltage relationship of the Ni\(^{2+}\)-sensitive current, representing \( I_{NCX} \) (n=9).

Figure 2: Cumulative I-V plots from cultured myocytes. Non-infected (A, n=6), NCX\( \Delta \)562-679-infected myocytes (B, n=9), and NCX\( \Delta \)562-679-infected myocytes with XIP 100\( \mu \)mol/L in the pipette solution (C, n=9). D) Comparison of the slope conductance (G, nS) between control (white bars) and Insulin treated cells (black bars) for all experimental groups including expression of wild-type canine NCX1.1 (WT NCX).

Figure 3: A) Cumulative I-V plots from fresh myocytes preincubated with L-NAME. Ins (1 \( \mu \)mol/L) induced a significant increase on both NCX modes in the presence of L-NAME (5mmol/L; n=8). B) The conductance (G) in the absence (white bars) or presence (black bars) of Ins when the cells were pretreated with L-NAME (right hand bars), compared with control cells (left hand bars). * indicates p<0.05 vs. control. The inhibition of the nitric oxide synthase with L-NAME (5mmol/L, n=8) did not prevent the insulin effect on both NCX modes.

Figure 4: A) Cumulative I-V plots from fresh myocytes pretreated for 30 minutes with 1mmol/L methyl-\( \beta \)-cyclodextrin (1mmol/L; n=9) A. B) The conductance (G) in the absence (white bars) or presence (black bars) of
Ins in fresh myocytes treated with MβCD (right hand bars) compared to controls (left hand bars). * indicates p<0.05 vs. control. Disruption of caveolae did not prevent the insulin effect on both NCX modes.

**Figure 5:** Cumulative I-V plots from fresh myocytes preincubated 2 hours with wortmannin (A, n=8) and with Chelerythrine (B, n=7). C) The conductance (G) in the absence (white bars) or presence (black bars) of Ins. * and ** indicate p<0.05 vs. the control in each group (without insulin) and vs. control without WM, respectively.

**Figure 6:** Ins effect in the presence of PIP2 in the pipette solution. Ins-effect on the average I-V relations in the presence of 30 μmol/L (A) or 60 μmol/L PIP2 (B). C) conductance in the absence (white bars) or presence (black bars) of Ins with different PIP2 concentrations. In A, B and C, * Indicates a significant difference (p<0.05) between Ins and control current in each condition. In C, ** indicates significant effect of PIP2 vs zero PIP2 control. Inset: the effect of PIP2 alone on NCX current (* indicates significant effect of PIP2 vs control).

**Figure 7:** Normalized insulin effect on NCX. A) The average increase in G_{NCX} induced by Ins (1μmol/L), expressed as % change from the control, examined in the presence of 1 mmol/L M-β-CD (n=9), 5 mmol/L L-NAME (n=8), 100 nmol/L WM (n=8) or 2.5 μmol/L Che (n=7). B) Bars compare the insulin effect on G_{NCX} in myocytes dialyzed with different PIP2 concentrations (30 or 60 μmol/L). C) Average increase in G_{NCX} in fresh myocytes, non-infected cultured myocytes, cultured myocytes expressing NCXΔ562-679 with or without XIP in the pipette solution and cultured myocytes expressing WT NCX. In all panels, * indicates that the insulin effect was statistically significant, ** indicates p<0.05 vs Ins alone (panel A and B) and vs. fresh or non-infected cultured myocytes.
Figure 2
Figure 3
Figure 4
Figure 5
Figure 7
Insulin effects on cardiac Na+/Ca2+ exchanger activity: role of the cytoplasmic regulatory loop
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