Protein Kinase C-Dependent Regulation of Na^+/Ca^{2+} Exchanger Isoforms NCX1 and NCX3 Does Not Require Their Direct Phosphorylation†

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ABSTRACT: We compared the phosphorylation-dependent regulation of three mammalian Na^+/Ca^{2+} exchanger isoforms (NCX1−NCX3) expressed in CCL39 fibroblasts that have little endogenous activity. Na^+_i-dependent $^{45}Ca^{2+}$ uptake into NCX1- or NCX3-expressing cells, but not that into NCX2-expressing cells, was significantly enhanced by phorbol 12-myristate 13-acetate (PMA) or platelet-derived growth factor-BB, which was abolished by pretreatment of cells with calphostin C or a prior long exposure to PMA. This suggests that NCX1 or NCX3, but not NCX2, is stimulated by a pathway involving protein kinase C (PKC). Immunoprecipitation experiments using [^{32}P]orthophosphate-labeled cells revealed that both NCX2 and NCX3 proteins were phosphorylated to a much lesser extent than the NCX1 protein in unstimulated cells and that the extent of phosphorylation was not increased by treatment with PKC activators, although NCX1 phosphorylation was enhanced significantly. Using site-directed mutagenesis, we identified three phosphorylation sites in the NCX1 protein in the PMA-stimulated cells to be Ser-249, Ser-250, and Ser-357 with Ser-250 being predominantly phosphorylated. We found that the NCX1 mutant with these serine residues substituted with alanine still maintained a normal response to PMA. In contrast, the NCX1 or NCX3 mutant, with the large central cytoplasmic loop deleted, lost the responsiveness to PMA. These results suggest that the PKC-dependent regulation of NCX1 or NCX3 requires the central cytoplasmic loop but does not require the direct phosphorylation of the exchanger.

The Na^+/Ca^{2+} exchanger of the plasma membrane is an electrogenic transporter that exchanges three Na^+ for one Ca^{2+}. The physiological importance of Na^+/Ca^{2+} exchange has been best studied in cardiomyocytes, where the exchanger has been shown to play the primary role in Ca^{2+} extrusion during the excitation−contraction cycle (*1*, *2*). In other cell types, the exchanger is also believed to extrude Ca^{2+} from the cytoplasm. The mammalian Na^+/Ca^{2+} exchanger forms a multigene family of homologous proteins comprising three isoforms, NCX1−NCX3 (*3*−*5*). These isoforms are ∼70% identical to one another in amino acid sequences, and have a similar deduced molecular topology consisting of 11 transmembrane-spanning segments and a large central hydrophilic loop. NCX1 is expressed to a great extent in cardiac muscle and to a lesser extent in many other tissues such as brain and kidney, whereas expression of NCX2 and NCX3 is significant only in brain and skeletal muscle (*4*, *5*).

Factors regulating Na^+/Ca^{2+} exchange in mammalian cells have been studied predominantly with NCX1. Previous studies employing electrophysiological and other techniques have shown that Na^+/Ca^{2+} exchange is activated by cytoplasmic Ca^{2+} (*6*, *7*) and external monovalent cations (*8*), but is inhibited by high cytoplasmic Na^+ concentrations (*7*, *9*), low cytoplasmic pH (*10*), and ATP depletion (*11*, *12*). Recent mutational analyses have revealed that a high-affinity Ca^{2+} binding site in the central cytoplasmic loop of NCX1 is required for the regulation by Ca^{2+} (*13*, *14*) and that the endogenous exchanger inhibitory peptide (XIP)[1] region is involved in the modulation by both intracellular Na^+ and Ca^{2+} (*15*). In cardiac giant patches, ATP has been shown to stimulate the exchange current by increasing the level of phosphatidylinositol 4,5-bisphosphate (*16*). Recent comparative studies of functions of NCX1−NCX3 expressed in mammalian cultured cells have shown that these isoforms have similar affinities for the extracellular transport substrates Na^+ and Ca^{2+} (*17*, *18*) but exhibit differential sensitivities to the inhibition by Ni^{2+} and other metal cations and isothiourea derivative KB-R7943 (*18*).

We have recently shown that exchange activity of NCX1 is stimulated by some growth factors and vasoactive agonists via a mechanism involving PKC in smooth muscle cells, cardiomyocytes, and NCX1-transfected cells (*12*, *19*). During such stimulation, the NCX1 protein is phosphorylated with a good correlation being observed between the activity and the extent of phosphorylation (*12*). There have been several other reports that suggest that NCX1 is regulated by protein phosphorylation (*17*, *20*−*23*). On the other hand, Linck et al. (*17*) have recently reported that exchange activity

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[1] Abbreviations: XIP, exchanger inhibitory peptide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PDGF-BB, platelet-derived growth factor-BB; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; BSS, balanced salt solution; Na^+_i, intracellular Na^+; Na^+_o, extracellular Na^+; Ca^{2+}_o, extracellular Ca^{2+}.

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of NCX3 expressed in BHK cells is enhanced by treatment with forskolin and inhibited by overnight treatment with PMA in a statistically significant manner. In invertebrates, the squid axon Na^+/Ca^{2+} exchanger, whose transport kinetics have been studied extensively, is known to be markedly stimulated by cytoplasmic ATP via a mechanism involving protein phosphorylation (see the references cited in ref *24*), whereas the frog cardiac Na^+/Ca^{2+} exchanger has recently been reported to be inhibited by β-adrenergic stimulation (*25*). These results provide strong evidence for the importance of protein phosphorylation in the modulation of Na^+/Ca^{2+} exchange, although the underlying mechanism still remains unclear.

In this study, we compared the phosphorylation-dependent regulation of NCX isoforms and investigated whether the direct phosphorylation of exchangers is required for the regulation by PKC activators. We found that exchange activity of NCX3, like that of NCX1, is upregulated by PKC activation, while activity of NCX2 is not. Such PKC-dependent regulation of NCX1 or NCX3 requires the large central cytoplasmic loop of the exchanger molecule, but does not require its direct phosphorylation.

EXPERIMENTAL PROCEDURES

Materials. $^{45}CaCl_2$ and [^{32}P]orthophosphate were obtained from Amersham. 8-Br-cAMP, 8-Br-cGMP, PMA, oligomycin, and BSA were purchased from Sigma. 4-α-PMA was from RBI. Calphostin C was from Kyowa Medex Co. Human PDGF-BB was from Pepro Tech. Octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$) was purchased from Nikkol Chemical Co. Ltd. (Tokyo, Japan). All other chemicals were of the highest grade available.

Cell Cultures. CCL39 cells (American Type Culture Collection) and NCX transfectants were maintained in Dulbecco's Modified Eagle's Medium supplemented with 7.5% heat-inactivated fetal calf serum, 50 units/mL penicillin, and 50 μg/mL streptomycin.

Cloning and Stable Expression of NCX1−NCX3 cDNAs. cDNAs of dog heart NCX1, rat brain NCX2, and rat brain NCX3 isolated as described previously (*12*, *18*) were subcloned into pCRII plasmids (Invitrogen) (designated pCRII-NCX1, pCRII-NCX2, and pCRII-NCX3, respectively). The cDNAs were inserted between *Sac*II and *Hin*dIII restriction sites of the mammalian expression vector pKCRH, and transfection of CCL39 cells was carried out as described previously (*12*). Cell clones exhibiting high Na^+/Ca^{2+} exchange activity were selected by treating colonies with 500 μg/mL G418 for 10 days and then with 10 μM ionomycin for 30 min. The ionomycin treatment ("Ca^{2+} killing") effectively eliminates cells with low exchange activity (*26*).

Construction of NCX Mutants. For construction of an NCX1 mutant with amino acids 244−671 deleted (numbers based on ref *27*), sense and antisense primers (5′-ATATCTCGAGAATTCAAGAGTACCGTGG-3′ and 5′-ATATCTCGAGCTCC TTCGTGCTCGATGA-3′) containing an exogenous *Xho*I restriction site (underlined) were used in combination with two outer primers corresponding to the 3′ (antisense) and 5′ noncoding (sense) regions, respectively. For construction of the NCX3 mutant with amino acids 292−708 deleted (numbers based on ref *5*), sense and antisense primers (5′-ATATGATATCTGCAGCAGGAGATGAGGA-3′ and 5′-ATATGATATCATTTTCCCATCCATCTCA-3′) containing an exogenous *Eco*RV restriction site (underlined) were used in combination with two outer primers corresponding to the 3′ (antisense) and 5′ noncoding (sense) regions, respectively. Using these pairs of primers, DNA fragments were generated by PCR with pCRII-NCX1 and pCRII-NCX3 as templates, digested with *Xho*I or *Eco*RV, and then inserted between *Sac*II and *Hin*dIII restriction sites of pKCRH. Successful construction was verified by sequencing (ABI PRISM, Perkin-Elmer).

Substitution of serine residues with alanine in NCX1 was achieved by site-directed mutagenesis using the "fusion PCR" approach (*28*). In this procedure, two DNA fragments were produced by PCR using *Pfu* polymerase and two pairs of outer and inner primers with the latter containing an overlapping sequence with the same desired mutation. The final PCR product was generated with these DNA fragments as templates using two outer primers. For mutations in amino acids 112−530, sequences for the outer sense and outer antisense primers were 5′-GGAGACCTAGGTCCC-AGCACC-3′ (the endogenous *Avr*II restriction site is underlined) and 5′-ATTTC CTCGAGCTCCAGATGT-3′ (the endogenous *Xho*I restriction site is underlined), respectively. For mutations in amino acids 534−828, the outer primers were 5′-TGGAGCTCGAGGAAATGTTAT-3′ (the endogenous *Xho*I restriction site is underlined) and 5′-TATG-GACGCGTCTGCATACTG-3′ (the endogenous *Mlu*I restriction site is underlined), respectively. *Avr*II−*Xho*I and *Xho*I−*Mlu*I fragments of the final PCR products were exchanged with the corresponding regions in pCRII-NCX1, and then the full-length mutant cDNAs were inserted into pKCRH. Successful construction was verified by sequencing.

Antibody Production. Polyclonal antibodies against NCX isoforms were raised by immunizing rabbits with maltose-binding protein fusion proteins or glutathione *S*-transferase fusion proteins containing either amino acids 240−737 of NCX1, amino acids 281−637 (numbers based on ref *4*) of NCX2, or amino acids 275−637 of NCX3. Preparation of the fusion proteins and affinity purification of the polyclonal antibodies were performed as described previously (*29*). Monoclonal antibodies were also raised by immunizing BALB/c mice with these fusion proteins using the method described previously (*30*). The monoclonal antibodies against the NCX1−NCX3 proteins (designated 6H3, 2S1, and 3U3, respectively) were purified from culture supernatants of positive hybridoma clones by protein A-Sepharose affinity chromatography. The isoform specificity of these polyclonal and monoclonal antibodies was verified by immunoblot analysis using membrane fractions prepared from cells expressing respective NCX isoforms (Figure 1).

Immunoprecipitation and Immunoblotting. Confluent cells expressing each NCX isoform in 100 mm dishes were labeled for 5 h at 37 °C in a phosphate-free, serum-free medium containing [^{32}P]orthophosphate (10 MBq/mL). NCX proteins were immunoprecipitated with respective rabbit polyclonal antibodies and subjected to SDS−PAGE on a 7.5% gel and then blotted to PVDF membranes, as described in detail previously (*12*). ^{32}P-labeled proteins on the blotting membranes were visualized by the Bioimage analyzer (BAS2000, Fuji Film Co.). The proteins on the same membranes were then immunostained with monoclonal antibodies raised

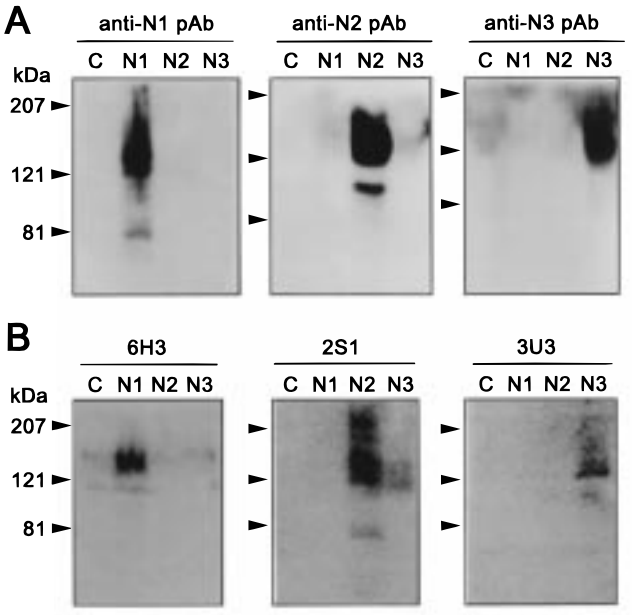

FIGURE 1: Isoform specificity of anti-NCX polyclonal (A) and monoclonal antibodies (B). Microsomes were prepared from parental CCL39 cells (C) and cells expressing NCX1 (N1), NCX2 (N2), or NCX3 (N3), and 30 μg of each of them was subjected to immunoblot analysis with anti-NCX polyclonal (pAb) and monoclonal antibodies. The NCX antibodies, except for 2S1, exhibited isoform-specific immunoreactivity to the respective NCX isoform. Anti-NCX2 antibody 2S1 cross-reacted slightly with the NCX3 protein. Molecular mass markers (in kilodaltons) are shown on the left.

against the NCX isoforms and visualized using an anti-mouse secondary antibody and the ECL detection system (Amersham). The densities for the ^{32}P incorporation and protein staining were quantified by AIS software (Imaging Research), and their ratio was calculated.

Phosphopeptide Mapping. After SDS−PAGE of the immunoprecipitated materials from ^{32}P-labeled cells, NCX proteins were eluted from homogenates of gel slices by overnight incubation in 50 mM NH_4HCO_3 (pH 8.0) containing 1% SDS, 1% 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride at 37 °C. Eluted proteins were precipitated with 10% trichloroacetic acid in the presence of BSA. Proteins were then washed with ice-cold acetone, resuspended in 500 μL of 50 mM NH_4HCO_3 (pH 8.0) containing 1 mM $CaCl_2$, and digested with 50 μg of tosylamide-2-phenylethyl chloromethyl keton-treated trypsin at 37 °C for 12 h. After lyophilization, trypsinized proteins were separated by thin-layer electrophoresis (KODAK chromagram sheet, pH 1.9, 1000 V, 30 min) in the first dimension and by thin-layer chromatography (3:15:10:12 acetic acid/butanol/pyridine/water) in the second dimension. Phosphopeptides were visualized by Bioimage analyzer.

Assay of Na^+_i-Dependent $^{45}Ca^{2+}$ Uptake. For cell Na^+ loading, confluent cells in 24-well dishes were incubated at 37 °C for 30 min in 0.5 mL of BSS [10 mM Hepes/Tris (pH 7.4), 146 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM glucose, and 0.1% BSA] containing 1 mM ouabain and 10 μM monensin. $^{45}Ca^{2+}$ uptake was then initiated by switching the medium to Na^+-free BSS (replacing NaCl with equimolar choline chloride) or to normal BSS, both of which contained 0.1 mM $^{45}CaCl_2$ (1.5 μCi/mL) and 1 mM ouabain. After a 30 s incubation, $^{45}Ca^{2+}$ uptake was terminated by washing cells four times with an ice-cold solution containing 10 mM Hepes/Tris (pH 7.4), 120 mM choline chloride, and 10 mM $LaCl_3$. Cells were then solubilized with 0.1 N NaOH, and aliquots were taken for determination of radioactivity and protein. $^{45}Ca^{2+}$ uptake rates in NCX1-, NCX2-, and NCX3-expressing cells were 15-, 6-, and 12-fold greater, respectively, in Na^+-free BSS than in normal BSS. The Na^+_i-dependent portions of these activities were considered to be the expression of Na^+/Ca^{2+} exchange activity.

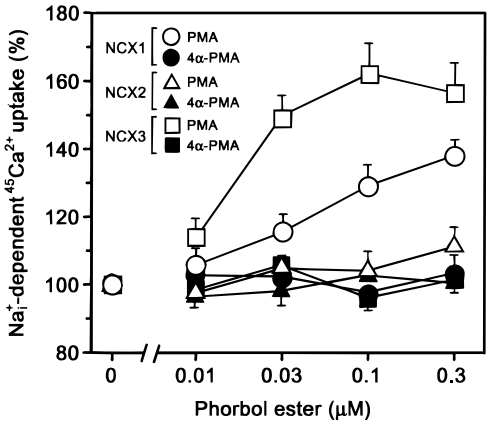

FIGURE 2: Dose-dependent effects of phorbol esters on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells expressing NCX1, NCX2, or NCX3. Cell Na^+ loading and measurement of Na^+_i-dependent $^{45}Ca^{2+}$ uptake were performed as described in Experimental Procedures. Uptake activities in the absence of phorbol esters were 11 ± 0.32, 3.7 ± 0.06, and 6.1 ± 0.12 nmol per milligram per 30 s (n = 3) in NCX1-, NCX2-, and NCX3-transfected cells, respectively, which are taken as 100% in the figure. Cells were treated with indicated concentrations of PMA or 4-α-PMA during the last 20 min of Na^+ loading. Data are means ± SE of three or four independent experiments.

Statistical Analysis. Data are expressed as means ± standard error of the mean (SE) of three or four independent determinations. Differences for multiple comparisons were analyzed by an unpaired t test or one-way ANOVA followed by the Dunnett's test. p values of <0.05 were considered statistically significant.

RESULTS

Regulation of Na^+_i-Dependent $^{45}Ca^{2+}$ Uptake into Cells Expressing NCX Isoforms. We used CCL39 fibroblasts for transfection of NCX cDNAs, because they expressed little endogenous Na^+/Ca^{2+} exchange activity (*12*) and very low or undetectable levels of NCX proteins (Figure 1 and ref *12*). We selected cells stably expressing high levels of NCX proteins by a Ca^{2+} killing procedure (see ref *26* and Experimental Procedures). In NCX1, NCX2, and NCX3 transfectants thus selected, the V_{max} values of Na^+_i-dependent $^{45}Ca^{2+}$ uptake, as determined from the Ca^{2+}_o concentration dependence of uptake activity, were 29 ± 2.6, 7.8 ± 0.7, and 16 ± 1.8 nmol per milligram per 30 s (n = 3), respectively.

We have recently shown that phorbol ester and some growth factors stimulate the rate of Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cardiomyocytes and NCX1-transfected CCL39 cells, while concomitantly enhancing phosphorylation of the NCX1 protein (*12*). Figure 2 shows the effects of PMA (0.01−0.3 μM) on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into NCX1,

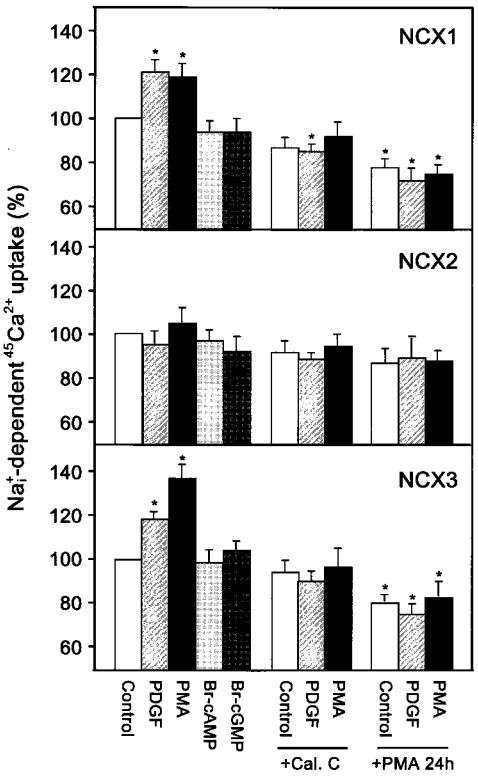

FIGURE 3: Effects of treatment with various protein kinase modulators and a long exposure to PMA on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells expressing NCX isoforms. Cells were treated with 100 ng/mL PDGF-BB, 0.1 μM PMA, 0.2 mM 8-Br-cAMP, or 0.2 mM 8-Br-cGMP during the last 20 min of Na^+ loading. In other series of experiments, cells were pretreated with 3 μM calphostin C (Cal. C) for 1 h or with 0.1 μM PMA for 24 h. Uptake activities in untreated cells are taken as 100%. Data are means ± SE of three or four independent experiments. The columns marked with an asterisk are significantly different from untreated control.

NCX2, and NCX3 transfectants. PMA accelerated Na^+_i-dependent $^{45}Ca^{2+}$ uptake into NCX1 and NCX3 transfectants up to 140 and 160% of control, respectively, whereas it minimally enhanced the uptake into NCX2 transfectants. On the other hand, 4-α-PMA, an inactive phorbol ester for PKC, exerted no effect on the uptake into all three transfectants. When the effect of 0.1 μM PMA on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into NCX3 transfectants was measured as a function of Ca^{2+}_o concentration (data not shown), the V_{max} of uptake activity increased by 42 ± 6% ($n = 3$) without a change in the K_m for Ca^{2+}_o (~0.2 mM). This mode of modulation by PMA was the same as that observed in NCX1 transfectants (*12*).

We further examined effects of PDGF-BB and some protein kinase modulators on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into NCX transfectants. PDGF-BB (100 ng/mL), like PMA, significantly enhanced the uptake in NCX1 and NCX3 transfectants, although this agent was less effective than PMA in NCX3 transfectants (Figure 3, top and bottom panels). Enhancement of the uptake by PDGF-BB or PMA was not observed when NCX1 and NCX3 transfectants had been treated with 0.3 μM calphostin C for 1 h or with 0.1 μM PMA for 24 h (resulting in PKC downregulation). Of note, the latter long PMA treatment itself caused a significant decrease (about 20%) in the uptake activity in unstimulated NCX1 or NCX3 transfetants (Figure 3). Thus, PKC is likely to be involved in the activation of both NCX1 and NCX3 in PDGF-BB-treated or unstimulated control cells. In contrast to these results, PDGF-BB, calphostin C, or long exposure to PMA did not exert a significant effect on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into NCX2 transfectants (Figure 3, middle panel). We also found that the uptakes into all these NCX transfectants were not affected by prior treatment with 0.2 mM 8-Br-cAMP or 0.2 mM 8-Br-cGMP for 20 min (Figure 3) or with 50 μM forskolin for 10 min (data not shown).

Phosphorylation of NCX Isoforms. We examined direct phosphorylation of NCX proteins in the [^{32}P]orthophosphate-labeled transfectants in response to various protein kinase activators (Figure 4). The NCX proteins were isolated from cell lysates by immunoprecipitation with isoform-specific antibodies, applied to SDS−PAGE, and then visualized by blot analysis. As described in Experimental Procedures, both the extent of ^{32}P label incorporation and the protein amount were estimated densitometrically using the same blot membranes. The NCX1 protein with a molecular mass of 120−140 kDa exhibited a significant basal phosphorylation level, which increased further to 146 ± 8 and 148 ± 11% ($n = 3$) of the control in response to 0.1 μM PMA and 100 ng/mL PDGF-BB, respectively, after the normalization by protein amount (Figure 4, top two panels). In sharp contrast to these results, the NCX2 and NCX3 proteins with molecular masses of 110−130 kDa exhibited only a slight basal phosphorylation level, and their phosphorylation level increased minimally (to 101−106% of the control) by treatment with PMA or PDGF-BB (Figure 4, middle two panels for NCX2 transfectants and bottom two panels for NCX3 transfectants). We found that 0.2 mM 8-Br-cAMP or 0.2 mM 8-Br-cGMP did not enhance phosphorylation of all NCX proteins (Figure 4). Thus, only the NCX1 isoform was directly phosphorylated in response to PMA or PDGF-BB.

Identification of Phosphorylation Sites in the NCX1 Protein. We showed previously that the extent of phosphorylation of NCX1 protein correlated well with the extent of activation of Na^+/Ca^{2+} exchange in cardiomyocytes as well as in NCX1 transfectants (*12*), suggesting the presence of a causative relationship between the two. To clarify the role of direct phosphorylation of the NCX1 protein, we identified the phosphorylation sites for PKC in this protein. Since multiple serine residues in the NCX1 protein were suggested to be phosphorylated by PKC (*12*), we introduced by site-directed mutagenesis serine to alanine mutations into nine candidate phosphorylation sites for PKC (Ser-66, Ser-249, Ser-250, Ser-325, Ser-357, Ser-466, Ser-467, Ser-529, and Ser-572) located in two putative cytoplasmic loops of the NCX1 protein. NCX1 mutants with one or more of these serine residues replaced by alanine were stably expressed in CCL39 cells, and their phosphorylation in PMA-treated, ^{32}P-labeled cells was examined. We found that S249A, S250A, and S357A mutants exhibited reduced levels of phosphorylation. S250A mutation caused a remarkable decrease in the phosphorylation level (to about 7% of that in wild-type NCX1) (Figure 5A, lane 4), whereas S249A or S357A mutation decreased it slightly (Figure 5A, lanes 3−6). Of note, triple mutation of these serine residues caused almost complete disappearance of the NCX1 phosphorylation (Figure 5A, lane 6). Thus, Ser-250 is the major PKC-dependent phosphorylation site in the NCX1 protein.

We further analyzed the phosphorylation of NCX1 mutants using two-dimensional tryptic phosphopeptide mapping

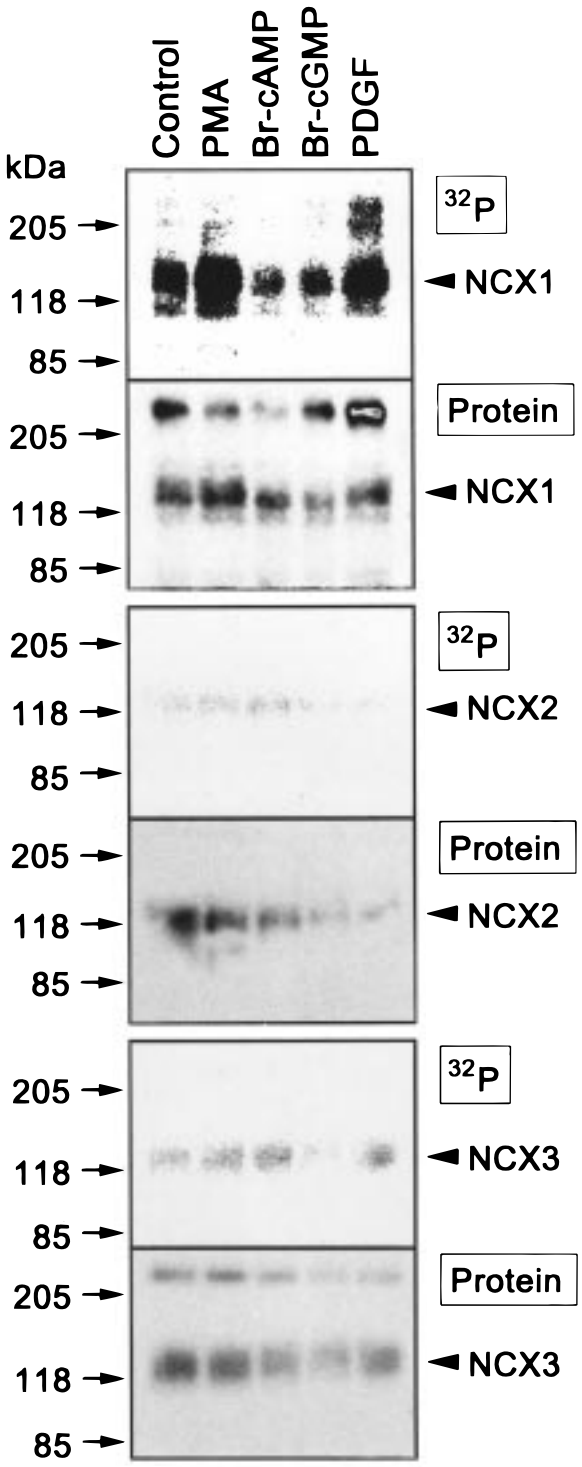

FIGURE 4: Phosphorylation of NCX1−NCX3 proteins in NCX transfectants. During the last 20 min of labeling with [^{32}P]-orthophosphate, cells were treated with 0.1 μM PMA, 0.2 mM 8-Br-cAMP, 0.2 mM 8-Br-cGMP, or 100 ng/mL PDGF-BB. NCX proteins were then isolated from cell lysates by immunoprecipitation with isoform-specific polyclonal antibodies. The immunoprecipitated materials were subjected to SDS−PAGE, transferred onto PVDF membranes, and visualized directly with a Bioimage Analyzer (upper panels) or by immunostaining with anti-NCX isoform monoclonal antibodies (lower panels). Molecular mass markers (in kilodaltons) are shown on the left.

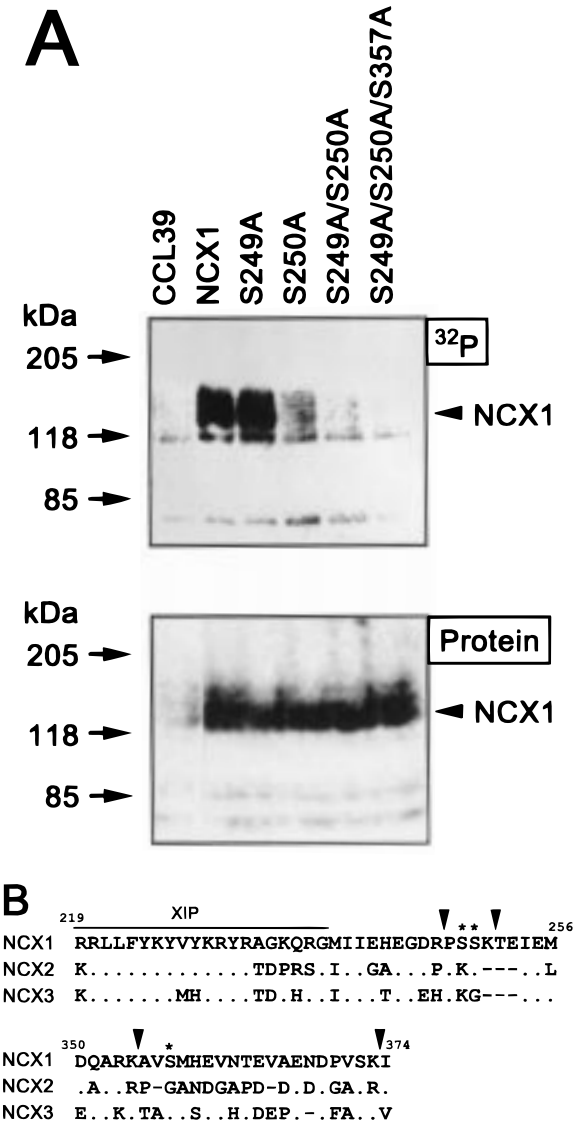

FIGURE 5: Phosphorylation sites of the NCX1 isoform. (A) Effects of serine to alanine substitutions on phosphorylation of the NCX1 protein. During the last 20 min of labeling with [^{32}P]orthophosphate, control cells and cells transfected with wild-type or mutated NCX1 cDNAs were treated with 0.1 μM PMA. The NCX1 protein was then immunoprecipitated from lysates of these cells, and the immunoprecipitated materials were subjected to SDS−PAGE and visualized with a Bioimage Analyzer (upper panel). From the same these cells, microsomes were prepared and 10 μg of each of them was subjected to immunoblot analysis with anti-NCX1 monoclonal antibody (lower panel). Molecular mass markers (in kilodaltons) are shown on the left. (B) Amino acid sequence of the region surrounding the phosphorylation sites in NCX1 and comparison with homologous regions in other NCX isoforms. Amino acid numbers are those of NCX1 (*27*). Identities between NCX1 and NCX2 or NCX3 are indicated with dots and alignment gaps with dashes. Three phosphorylatable serine residues are marked by asterisks. Arrowheads indicate trypsin digestion sites which could generate tryptic phosphopeptides from the NCX1 protein. The XIP region is shown by a horizontal line.

(Figure 6). Tryptic digestion of the ^{32}P-labeled wild-type NCX1 protein reproducibly generated three major phosphopeptides (designated P1−P3; see the lower right panel in Figure 6) whose contents were usually in the order of P1 > P2 > P3. Occasionally, some minor spots, which may represent partially digested phosphopeptides, were observed. The S250A mutation eliminated both P1 and P2 (Figure 6, middle left panel), whereas individual S249A and S357A mutations caused the disappearance of P2 and P3, respectively (Figure 6, upper and middle right panels). The S249A/S250A double mutation eliminated both P1 and P2 (data not

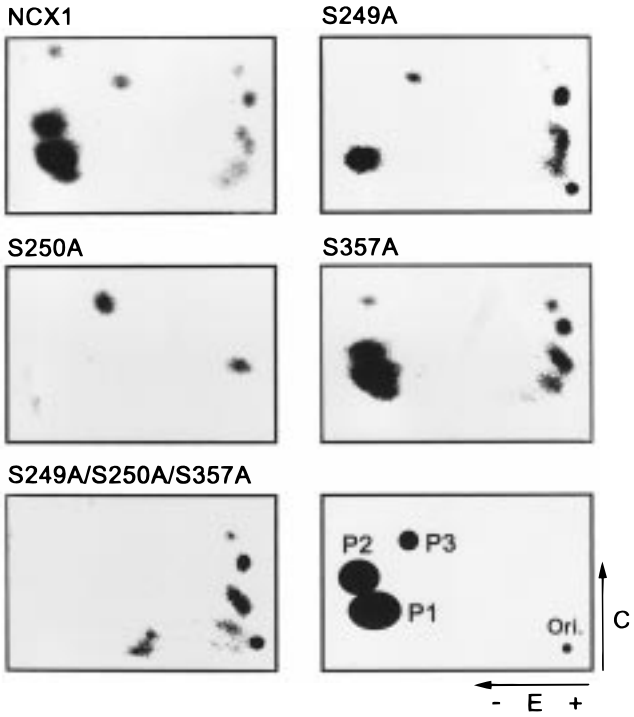

FIGURE 6: Two-dimensional tryptic phosphopeptide mapping of NCX1 mutants. Cells expressing the wild-type or mutated NCX1 were treated with 0.1 μM PMA for 20 min. Phosphorylated NCX1 proteins were isolated from these cells by immunoprecipitation and subsequent SDS−PAGE, and subjected to two-dimensional tryptic phosphopeptide mapping. The right bottom panel schematically shows the location of phosphopeptides and the direction of peptide migration (arrows).

shown), while the S249A/S250A/S357A triple mutation caused the disappearance of all three phosphopeptides (Figure 6, lower left panel). We interpreted these results as an indication that P1 and P3 contain phosphoserine at positions 250 and 357, respectively, while P2 contains phosphoserines at positions 249 and 250 (see the Discussion; see also Figure 5B for the phosphorylation site sequences).

Regulatory Properties of NCX Mutants. To assess the role of direct phosphorylation of the NCX1 protein in the PKC-dependent regulation, we examined the effect of PMA on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells expressing NCX1 mutants whose phosphorylatable serine residues were mutated to alanines. As shown in Figure 7, PMA enhanced the uptake by the S249A/S250A/S357A triple mutant in a dose−response manner similar to that for the wild-type NCX1, indicating that exchange activity of this mutant is regulated normally by PKC. We obtained similar results with single mutants S249A, S250A, and S357A (data not shown). In contrast, the PMA-induced activation of uptake activity was not observed in a mutant (Δ246−672) with most of the central cytoplasmic loop of NCX1 deleted (Figure 7). Similar deletion mutation (Δ292−708) in NCX3 also resulted in the loss of the PMA-induced activation of uptake activity (Figure 7). These results suggest that the central cytoplasmic loop is required for the PKC-dependent regulation of exchange by NCX1 or NCX3.

Cell ATP depletion is known to inhibit Na^+/Ca^{2+} exchange in several cell types (*11*, *12*, *17*, *31*), although its underlying mechanism remains unclear. We studied the effect of a 10 min treatment with 2.5 μg/mL oligomycin and 10 mM

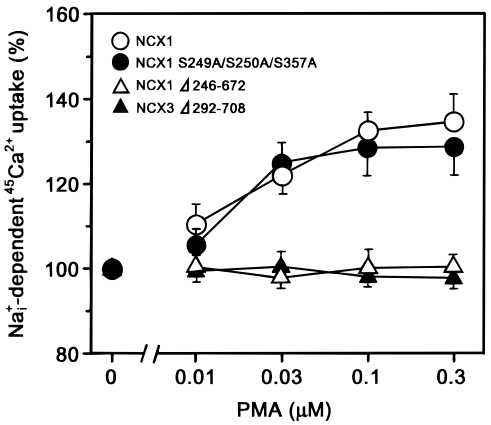

FIGURE 7: Dose-dependent effects of PMA on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells expressing the wild-type NCX1, the triple serine to alanine NCX1 mutant, and the mutants of NCX1 and NCX3 with the large cytoplasmic loop deleted. Na^+_i-dependent $^{45}Ca^{2+}$ uptake rates were measured as described in the legend of Figure 2. Uptake rates in the absence of PMA were 10 ± 0.21, 8.7 ± 0.63, 4.6 ± 0.11, and 3.6 ± 0.08 nmol per milligram per 30 s (n = 3) in cells expressing the wild-type NCX1, the S249A/S250A/S357A NCX1 mutant, NCX1Δ246−672, and NCX3Δ292−708, respectively. Uptake activities in the absence of PMA are taken as 100%. Data are means ± SE of three independent experiments.

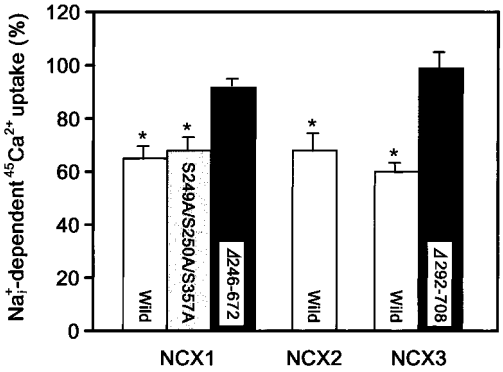

FIGURE 8: Effects of ATP depletion on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells expressing wild-type NCX isoforms, the triple serine to alanine NCX1 mutant, NCX1Δ246−672, and NCX3Δ292−708. Cell Na^+ loading and measurement of the Na^+_i-dependent $^{45}Ca^{2+}$ uptake rates were performed as described in Experimental Procedures. The cells had been incubated with 10 mM 2-deoxy-D-glucose and 2.5 μg/mL oligomycin or with 10 mM D-glucose (control) during the last 10 min of Na^+ loading. Average uptake activity in untreated control cells is taken as 100% for each cell clone. Data are means ± SE of three independent experiments. The columns marked with an asterisk are significantly different from the control.

2-deoxy-D-glucose on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into CCL39 cells expressing wild-type or mutated NCX isoforms (Figure 8). This treatment reduced the uptake rate by the wild-type NCX1 or the S249A/S250A/S357A NCX1 triple mutant to a similar extent (to 60−70% of the control), indicating no involvement of the direct phosphorylation of NCX1 protein in the observed inhibition of uptake activity. We found that these metabolic inhibitors similarly inhibited the uptake by wild-type NCX2 or NCX3 (Figure 8). Interestingly, however, the inhibition by these agents was not observed in NCX1Δ246-672 or NCX3Δ292-708, suggesting that the central cytoplasmic loop is involved in the ATP-dependent inhibition of exchange by NCX1 or NCX3.

This observation obtained with the NCX1 mutant lacking the central cytoplasmic loop is consistent with a previous report (*11*).

DISCUSSION

In our recent study (*12*, *19*), we have provided evidence that transport activity of NCX1 is stimulated by PKC activation in cultured smooth muscle cells, cardiomyocytes, and NCX1-transfected CCL39 cells. The aim of this work is to compare phosphorylation-dependent regulation of three mammalian NCX isoforms and to obtain insight into the mechanisms involved. To characterize individual NCX isoforms in the same cellular background, we stably expressed each in CCL39 fibloblasts that exhibit little endogenous activity (see the Results and ref *12*).

This study has shown that Na^+_i-dependent $^{45}Ca^{2+}$ uptake into NCX3 transfectants, like that in NCX1 transfectants, was enhanced by a short treatment with PMA, but significantly inhibited by a long exposure to PMA, a procedure known to cause downregulation of PKC in cells (Figures 2 and 3). PDGF-BB also enhanced uptake activities in NCX1 and NCX3 transfectants, which were abolished by treatment with calphostin C or by a long exposure to PMA. In contrast, Na^+_i-dependent $^{45}Ca^{2+}$ uptake into NCX2 transfectants was not influenced by all these procedures, indicating that NCX2 differs from NCX1 or NCX3 in the regulatory response to PKC activation. When the kinetics of Na^+_i-dependent $^{45}Ca^{2+}$ uptake were measured as a function of Ca^{2+}_o concentration, PMA was found to increase the V_{max} without affecting the K_m for Ca^+_o in NCX3 transfectants (see the Results), which is similar to the finding obtained with NCX1 transfectants (*12*). It is unlikely that such an increase in the V_{max} was caused by an increase in the number of exchanger molecules in the plasma membrane due to their cellular redistribution induced by PMA, because the number of c-myc epitopes in the plasma membrane of CCL39 cells expressing the c-myc epitope-tagged NCX1 protein was not affected by a 30 min treatment with PMA, as estimated immunologically (*32*) (T. Iwamoto et al., unpublished observation). In contrast to the effect of PMA or PDGF-BB, 8-Br-cAMP, forskolin, or 8-Br-cGMP did not affect Na^+_i-dependent $^{45}Ca^{2+}$ uptake into all these NCX transfectants (Figure 3 and Results). Because a previous study provided clear evidence that the activation status of cAMP-dependent protein kinase closely follows the level of intracellular cAMP in CCL39 cells transfected with muscarinic M1 receptor cDNA (*33*), it appears that transport activities of all NCX isoforms are not influenced by cAMP-dependent protein kinase in the CCL39 cell line. This result is at variance with the finding by Linck et al. (*17*), who found that exchange activities of NCX1 and NCX3 expressed in BHK cells are stimulated by treatment with forskolin. Currently, we have no explanation for this discrepancy.

Previously, we showed that the NCX1 protein was phosphorylated in cultured smooth muscle cells, cardiomyocytes, and NCX1-transfected CCL39 cells during the stimulation of exchange activities by PKC activators (*12*, *19*). We found that the extent of phosphorylation of the NCX1 protein correlated well with the extent of activation of exchange activity in these cells, which suggests the presence of a causative relationship between the two (*12*). We also found that okadaic acid, a specific inhibitor of protein phosphatases 1 and 2A, enhanced both exchange activity and phosphorylation of the NCX1 protein in cardiomyocytes and NCX1 transfectants (*12*). This study confirmed the significant phosphorylation of the NCX1 protein in unstimulated transfectants and its enhancement in response to stimuli that activate PKC (Figure 4). In addition, we showed for the first time that NCX2 and NCX3 proteins are phosphorylated to a much lesser extent than the NCX1 protein in unstimulated transfectants and that their phosphorylation was not influenced by treatments with various PKC modulators as well as with 8-Br-cAMP or 8-Br-cGMP (Figure 4). The finding that NCX3 is not phosphorylated in response to PKC activators suggests that direct phosphorylation of the exchanger protein is not required for the PKC-dependent enhancement of exchange activity at least in NCX3 transfectants.

To clarify the regulatory role of direct phosphorylation of the NCX1 protein, we identified the phosphorylation sites in PMA-treated, [^{32}P]orthophosphate-labeled NCX1 transfectants using site-directed mutagenesis. Since multiple serine residues were previously shown to be phosphorylated in the NCX1 protein (*12*), nine serine residues localized in putative intracellular loops were selected as candidate PKC phosphorylation sites [R/K-$(X)_{0-2}$-S] (*34*) and then mutated to alanines. Immunoprecipitation experiments revealed that Ser-250 is the predominant phosphorylation site, with Ser-249 and Ser-357 being phosphorylated to a much lesser extent (Figure 5A). In two-dimensional phosphopeptide mapping, tryptic digestion of the ^{32}P-labeled wild-type NCX1 protein generated three major phosphopeptides, P1−P3 (Figure 6). When cells expressing mutants with single, double, or triple mutants of these phosphorylatable sites were subjected to phosphopeptide mapping, P1 and P3 were missing from all mutants carrying the S250A or S357A mutation, respectively, suggesting that P1 and P3 contain phosphoserine-250 and phosphoserine-357, respectively (cf. Figure 5B). On the other hand, P2 disappeared in mutants carrying the S249A or S250A single mutation, the S249A and S250A double mutation, or the S249A/S250A/S357A triple mutation (Figure 6). We interpreted these results as indicating that adjacent Ser-249 and Ser-250 are both phosphorylated in P2, on the basis of the assumptions that P2 from the S249A mutant with its Ser-250 phosphorylated in response to PMA exhibits an electrophoretic mobility similar to that of P1 (containing phosphoserine-250) and that Ser-249 is phosphorylated only after the phosphorylation of Ser-250. The latter assumption is required to explain the disappearance of both P1 and P2 in the S250A mutant (Figure 6). The postulated sites for tryptic cleavage and sequences of the phosphopeptides that are produced are depicted in Figure 5B. Interestingly, these three phosphorylatable sites are not conserved in NCX2 or NCX3, consistent with the phosphorylation data obtained with these isoforms.

We examined the effect of PMA on Na^+_i-dependent $^{45}Ca^{2+}$ uptake into cells expressing the NCX1 mutant carrying the S249A/S250A/S357A triple mutation. We found that this mutant still maintained a normal response to PMA (Figure 7), suggesting that direct phosphorylation of NCX1 protein is not required for the upregulation of exchange activity by PKC activation. In contrast, the NCX1 mutant (NCX1Δ246−

672), with most of the large central cytoplasmic loop deleted, did not exhibit PMA-induced activation of exchange activity (Figure 7). A similar loss of the regulation by PMA was observed in the NCX3 mutant (NCX3Δ292−708) with the central cytoplasmic loop deleted. Of note, these NCX1 and NCX3 deletion mutants still retained 53 and 59%, respectively, of uptake activities of the corresponding wild-type exchangers (see the legend of Figure 7). These results suggest that the large central cytoplasmic loop is required for the PKC-dependent regulation of NCX1 or NCX3. This requirement of the central cytoplasmic loop of the exchanger does not support the view that the effect of PKC on exchange activity is secondary to its actions on other cellular systems such as the Na^+ pump that could alter ion distribution across the cell membrane. Such an indirect effect, if it occurs, should have caused a similar upregulation of exchange activities of the NCX1 and NCX3 mutants with deletion of the central cytoplasmic loop as well as of the activity of the wild-type NCX2.

The data discussed in the preceding paragraphs suggest that the PKC-dependent regulation of NCX1 and NCX3 is very similar with respect to the kinetic effect and the molecular mechanism. The requirement of the central cytoplasmic loop for their regulation and the absence of requirement of the direct phosphorylation of exchanger proteins suggest the involvement of regulatory protein(s), which may exist in the cytoplasm transmitting signals generated by PKC activators to the NCX1 and NCX3 molecules. In this context, it is very interesting to note that a novel 13 kDa cytosolic protein, possibly involved in the phosphorylation-dependent regulation of the squid exchanger, has recently been isolated from the axoplasm and brain of squid (*35*). To date, there has been some controversy with regard to the importance of protein phosphorylation in the regulation of Na^+/Ca^{2+} exchange. Some workers did not obtain evidence for the involvement of protein kinases in the NCX1 regulation (*11*, *36*), whereas others obtained data supporting the view that NCX1 activity is regulated by a phosphorylation-dependent mechanism (*12*, *17*, *19*−*23*). Such inconsistency could arise partly from the variable content of the putative regulatory factor(s) in the cell or membrane preparations used in these studies.

The role of direct phosphorylation of the NCX1 protein induced by PKC activators is not clear at present. It should be noted that the major phosphorylation site (Ser-250) exists immediately C-terminal to the autoinhibitory XIP region (see Figure 5B) that is known to be involved in the Na^+_i-dependent inactivation and Ca^{2+}-dependent regulation of NCX1 activity (see the introductory section). It is thus possible that phosphorylation of Ser-250 may modulate these regulations in NCX1. Alternatively, direct phosphorylation of the NCX1 protein could be involved in some other unknown functions of this protein that are modulated by PKC activation. In this study, we found that ATP depletion-induced inhibition of exchange activity occurs similarly in the S249A/S250A/S357A triple mutant of NCX1 and the wild-type NCX1 (Figure 8), suggesting that the direct phosphorylation is not involved in the inhibition by ATP depletion.

In summary, these results suggest that transport activities of NCX1 and NCX3 proteins are regulated by PKC in a manner not involving direct phosphorylation of these exchangers. In contrast, activity of NCX2 in CCL39 cells is not regulated by a pathway involving PKC. This regulatory difference between isoforms, which is also found in other ion transporters (*37*, *38*), could provide an important physiological mechanism by which the multiplicity of protein functions can be generated.

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