Protein Kinase C-dependent Enhancement of Activity of Rat Brain NCKX2 Heterologously Expressed in HEK293 Cells

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Different members of the Na+/Ca2++K+ exchanger (NCKX) family are present in distinct brain regions, suggesting that they may have cell-specific functions. Many neuronal channels and transporters are regulated via phosphorylation. Regulation of the rat brain NCKXs by protein kinases, however, has not been described. Here, we report an increase in NCKX2 activity in response to protein kinase C (PKC) activation. Outward current of NCKX2 heterologously expressed in HEK293 cells was enhanced by β-phorbol dibutyrate (PDBu), whereas PDBu had little effect on activity of NCKX3 or NCKX4. The PDBu-induced enhancement (PIE) of NCKX2 activity was abolished by PKC inhibitors and significantly reduced when the dominant negative mutant of PKCe (K437R) was overexpressed. Moreover, PDBu accelerated the decay rate of the Ca2++ transient at the calyx of Held, where NCKX is the major Ca2+-clearance mechanism. Intracellular perfusion with alkaline phosphatase completely inhibited PIE. Consistently, β-phorbol myristate acetate (PMA), but not 4α-PMA, induced a 3-fold stimulation of 32P incorporation into NCKX2 expressed in HEK293 cells. To investigate the sites involved, PIE of wild-type NCKX2 was compared with mutant NCKX2 in which the three putative PKC consensus sites were replaced with alanine, either individually or in combination. Double-site mutation involving Thr-476 (T166A/T476A and T476A/S504A) disrupted PIE, whereas single mutation of Thr-166, Thr-476, or Ser-504 or the double mutant T166A/S504A failed to completely prevent PIE. These findings suggest that PKC-mediated activation of NCKX2 is sensitive to mutation of multiple PKC consensus sites via a mechanism that may involve several phosphorylation events.

The family of Na+/Ca2++K+ exchangers (NCKX) catalyzes electrogenic exchange of four Na+ for one Ca2+ and one K+ across the plasma membrane (1–3). Although NCKX expression was originally considered to be restricted to retinal photoreceptors, new members of the NCKX family were identified from the brain and other excitable tissues (4–7). In the brain, transcripts for NCKX were preferentially detected in neurons rather than glia (8), implying that NCKX is involved in Ca2++ homeostasis specifically required for neurons. Indeed, it has been reported that NCKX plays a crucial role in Ca2++ extrusion from axon terminals of mammalian central neurons (9–11), and in Ca2++ homeostasis of cortical neurons (12, 13). Recently, impairment of synaptic plasticity in hippocampus, together with deficits in motor learning and spatial working memory, were found in NCKX2-null mice (14). Despite the importance of NCKX in the Ca2++ homeostasis in central nervous system neurons, little is known regarding its regulation.

Regulation of the cardiac-type Na/Ca exchanger, NCX1, has been extensively studied. NCX1 has been shown to be regulated by Ca2+, Na+, H+, the phospholipid environment of plasma membrane (15), and the action of protein kinases (16). Many proteins related to phosphorylation can be co-immunoprecipitated with NCX1, including protein kinases, phosphatases, and A-kinase anchoring proteins. It was suggested that NCX1 and these signaling molecules comprise a macromolecular complex (17–19). The large, central intracellular loop of NCX1 is the principal site of regulation (20) and has been suggested as the most probable anchoring site for other signaling molecules. Although there is little amino acid sequence identity between NCX and NCKX family members, they do share a similar predicted membrane topology. The NCX protein is proposed to consist of 11 transmembrane segments (TMs) and a large intracellular loop between TM5 and TM6 (21, 22). Moreover, the amino acid sequence of the NCKX2 intracellular loop contains a number of predicted motifs that may serve as phosphorylation sites for protein kinase A, protein kinase C (PKC), and Ca2+-calmodulin-dependent protein kinase II.
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Previously, we have shown that NCKX plays a major role in Ca\(^{2+}\) extrusion at the fast glutamatergic presynaptic terminal, calyx of Held (11). It is well known that phorbol esters enhance synaptic transmission by a presynaptic mechanism, which includes an enhancement of Ca\(^{2+}\) sensitivity of the molecular machinery that mediates synaptic vesicle fusion and an increase in the size of the readily releasable pool (23). The effect of phorbol esters on the presynaptic Ca\(^{2+}\) dynamics, however, still remains to be elucidated. Given that Ca\(^{2+}\) plays a key role in the activity-dependent changes of synaptic strength as well as in release of neurotransmitters, PKC modulation of NCKX would be expected to have substantial influence on presynaptic Ca\(^{2+}\) dynamics, and in turn on short-term synaptic plasticity.

In the present study, we have analyzed the regulation of neuronal NCKX family members by PKC. We found that PKC activation phosphorylated and activated NCKX2 expressed in HEK293 cells, whereas the activity of NCKX3 and NCKX4 was not affected. By using pharmacological intervention and mutagenesis of potential PKC phosphorylation sites, we have shown that Thr-476 as well as Thr-166 and Ser-504 are important residues involved in PKC-mediated activation of NCKX2.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human embryonic kidney (HEK293) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin in 5% CO\(_2\) at 37 °C. The cells were cotransfected with rat brain NCKX2 cDNA and DS-Red2 cDNA using a standard calcium-phosphate precipitation protocol with HBS buffer (140 mM NaCl, 0.75 mM Na\(_2\)HPO\(_4\), 25 mM Na\(_2\)EDTA, 25 mM HEPES, pH 7.0). The cells were used for electrophysiological recordings 36–48 h after the co-transfection.

Electrophysiology—Electrophysiological recordings were performed using the conventional whole cell configuration of the patch clamp recording technique. Patch pipettes with a resistance of 4–5 MΩ were prepared from borosilicate glass capillaries, which were filled with a pipette solution (see below). Voltage clamp experiments were conducted with an EPC-8 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). The holding potential was set to 0 mV. The membrane current was filtered at 500 Hz by a built-in low-pass filter, sampled at 1 kHz, and stored on a PC. The raw current recordings were low-pass filtered off-line using a boxcar smoothing algorithm with a smoothing factor of 5. The capacitive component of the current was minimized by the cancellation circuit built in the voltage clamp amplifier. Voltage ramp pulses were applied before and after evoking NCKX2 current using perfusion solution changes (see below). The current-voltage (I-V) relationship was obtained from the current response to a ramp pulse of amplitude from −80 mV to +80 mV over 200 ms. For clarity, in Fig. 7B and supplemental Fig. S2B, slow \(I_{\text{NCKX2}}\) traces are presented after removing artifacts caused by voltage ramp pulses. All experiments were performed at room temperature (24 ± 1 °C).

Solutions—To record reverse-mode (outward) NCKX-exchange currents, we used a pipette solution containing 120 mM NaCl, 20 mM tetraethylammonium-Cl, 10 mM BAPTA, 20 mM HEPES, 4 mM Mg-ATP (pH 7.2 adjusted with NaOH). Current recordings in control conditions (where NCKX2 was inactive) were obtained while the cells were bath-perfused with a Ca\(^{2+}\)- and K\(^{+}\)-free bath solution containing 120 mM LiCl, 0.5 mM EGTA, 1 mM MgCl\(_2\), 20 mM tetraethylammonium-Cl, 20 mM HEPES, 10 mM glucose, and pH 7.4 adjusted with LiOH (this solution is referred to as “Li solution” under “Results”). Reverse-mode Na\(^{+}/Ca^{2+}/K^{+}\) exchange was induced by bath-application of a test solution, which was composed of 120 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 20 mM tetraethylammonium-Cl, 20 mM HEPES, 10 mM glucose, and pH 7.4 adjusted with tetramethylammonium-OH (referred to as “Ca/K solution”).

Site-directed Mutagenesis—Site-directed mutagenesis was carried out using the GeneTailer site-directed mutagenesis kit (Invitrogen) according to the manufacturer’s protocol with appropriate pairs of complementary mutagenic primers. Mutants were constructed from the FLAG-tagged rat brain NCKX2 (fNCKX2) (4) in the pcDNA3.1 vector. Single or double point mutations were created to replace serine or threonine residues with alanine. All constructs were verified by DNA sequencing prior to use.

Dominant Negative Mutations of PKCs—The expression vector pHACE (24) was used to generate plasmids that encode the dominant negative mutant of PKC with a C-terminal hemagglutinin tag. pHACE-PKC-DN expression plasmids were generated by ligating full-length open reading frames of PKC isoforms with a dominant negative (DN) point mutation at the ATP binding site (K368R, K376R, K437R, and K384R for PKCo, PKCβ, PKCe, and PKCd, respectively) into pHACE digested with EcoRI (see Ref. 24 for details). The expression of each PKC-DN in HEK293 cells was confirmed by Western blot analysis using anti-HA antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA). An anti-tubulin blot was performed as a loading control (anti-tubulin antibody, Sigma). Relative expression of PKC-DN mutants was determined by comparative densitometry of Western blots (supplemental Fig. S1).

Photometry—Two days after transfection, HEK293 cells on poly-d-lysine-coated cover slips were loaded with 5 μM fura-2 AM (Molecular Probes) and mounted in a perfusion chamber on a microscope stage. The fura-2 ratio was determined on a field of cells by measuring fluorescence from excitation at 340 nm and 380 nm with a D-104 microscope photometer using Felix version 1.42 software (Photon Technology International). The perfusion solutions used contained either 140 mM NaCl or LiCl together with 5 mM KCl, 0.1 mM CaCl\(_2\), 10 mM D-glucose, and 10 mM HEPES-tetramethylammonium (pH 7.4). The cells were perfused alternatively for 3 min with sodium buffer or 2 min with lithium buffer. Half way through the second perfusion with sodium buffer, the cells were incubated for 15 min with either 0.1 μM, β-phorbol myristate acetate (PMA), or 4α-PMA. Ca\(^{2+}\)-transport rates for reverse-mode NCKX2 activity were determined by linear regression of the initial linear rate of change of the fura-2 ratio using GraphPad Prism version 4.0 software.
**In Vivo Phosphorylation Assay**—Two days after transfection, HEK293 cells in 100-mm dishes were washed twice with 5 ml of Dulbecco’s modified Eagle’s medium without sodium phosphate and then incubated with 100 µCi/ml 32P-labeled orthophosphate in 2 ml of Dulbecco’s modified Eagle’s medium without sodium phosphate for 3–4 h at 37 °C. The cells were then treated with or without 1 µM ionomycin and/or 0.1 µM PMA or 4µM-PMA for 15 min at room temperature. The cells were then placed on ice, washed twice with 5 ml of ice-cold phosphate-buffered saline and lysed in situ with 1 ml of IP buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 1% Triton X-100, 120 mM NaCl, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 100 units/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor tablets (Roche Applied Science)). All subsequent steps were performed at 4 °C. The lysate was transferred to a microcentrifuge tube and incubated on ice for 20 min followed by centrifugation at 14,000 rpm for 5 min. 25 µg of protein extract was frozen at −80 °C for later SDS-PAGE and immunoblotting analysis. 5 mg of protein extract from the supernatant was adjusted to 1 ml with the IP buffer, precleared with protein A-Sepharose beads and transferred to a new tube. The supernatant was mixed with the IP buffer, precleared with protein A-Sepharose beads and transferred to a new tube. The supernatant was mixed with 5 µg of M2 anti-FLAG monoclonal antibody by rotating for 2 h followed by addition of 80 µl of 25% protein A beads for 30 min. The beads were washed three times in IP buffer by centrifuging at 3,000 rpm for 2 min. The sample was eluted by adding 40 µl of 4× SDS sample buffer containing 8% 2-mercaptoethanol and heating to 50 °C for 5 min.

The immunoprecipitated samples and protein extracts were resolved on 9% SDS-PAGE gels and transferred to nitrocellulose membranes. The 32P autoradiograph was obtained by incubating the membranes with x-ray film in a cassette containing one intensifying screen at −80 °C for 3–48 h. No signal was observed after 15 min of incubation.

The membranes were subsequently incubated in phosphate-buffered saline containing 0.1% Tween 20, 5% skim milk powder and probed with the affinity-purified anti-NCKX2 polyclonal antibody F (21), followed by application of horseradish peroxidase-conjugated anti-rabbit IgG antibody. The membranes were developed using ECL reagents and typically required exposure times of 1 s to 2 min. The band intensities were quantified using ImageJ software (National Institutes of Health), and 32P incorporation was determined by dividing the required exposure times of 1st o2min. The band intensities observed after 15 min of incubation.

**RESULTS**

**Na⁺/Ca²⁺+K⁺ Exchange Currents (I_{NCKX2}) in Rat NCKX2-transfected HEK293 Cells**—Using whole cell patch clamp technique, outward NCKX2 current was recorded at a holding potential of 0 mV from NCKX2-transfected HEK293 cells, which were internally dialyzed with a high concentration of NaCl (2). The reverse Na⁺ gradient favors Ca²⁺ entry in the presence of extracellular Ca²⁺ and K⁺. To prevent the rapid increase in cytosolic [Ca²⁺], which was expected to develop as a consequence of exchange in reverse mode, the pipette solution contained 10 mM BAPTA. The control bathing solution contained LiCl and EGTA, which provides no counterpart to be exchanged for Na⁺. NCKX2-transfected cells generated outward current at 0 mV when they were exposed to bath solution containing both Ca²⁺ and K⁺ together, but not with either alone (Fig. 1A, left). I_{NCKX2} was not significantly affected by 0.5 mM ouabain, indicating that the Na⁺ pump current did not contaminate our data (not shown). When non-transfected HEK293 cells were analyzed under identical conditions, they produced no obvious current (Fig. 1A, right). To obtain current-voltage (I-V) relationships, the cells were subjected to voltage ramps ranging from −80 to +80 mV during the bath perfusion of control or test solutions. The I-V curve observed from the NCKX2-transfected cell during the bath-perfusion of Ca/K solution showed no reversal, which was clearly distinct from the control I-V curve, indicating that the outward current corresponded to the electrogenic movement of Na⁺ in exchange for Ca²⁺ and K⁺ catalyzed by the NCKX2 protein.
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PDBu Enhanced \( I_{\text{NCKX2}} \) in a Dose-dependent Manner—When outward \( I_{\text{NCKX2}} \) was elicited repeatedly by alternating bath application of Ca/K solution (for 15–20 s, every 2 min) with Li solution, spontaneous enhancement of outward \( I_{\text{NCKX2}} \) was never observed in control conditions (Fig. 2A, left). In the presence of 100 nM PDBu, however, outward \( I_{\text{NCKX2}} \) was significantly and progressively enhanced (Fig. 2B, left). The enhancement started as early as 2 min after the bath application of PDBu. The I-V relationship of outward currents in the control condition and in the presence of PDBu shows that PDBu enhanced the outward current uniformly over the entire voltage range tested (from \(-80 \text{ mV} \) to \(+80 \text{ mV} \)), and thus no reversal was observed between the two I-V curves, indicating that PDBu did not activate endogenous nonselective cationic current (Fig. 2B, right). The time course for the change in the amplitude of \( I_{\text{NCKX2}} \) at different concentrations of PDBu is shown in Fig. 2C. The dose dependence of the PDBu effect on \( I_{\text{NCKX2}} \) was quantitated as the peak amplitude of \( I_{\text{NCKX2}} \) measured at a given time following the application of PDBu, normalized to the averaged amplitude of two or three \( I_{\text{NCKX2}} \) peaks elicited before the drug application from the same cell (this estimate is referred to below as “relative amplitude of \( I_{\text{NCKX2}} \)”). PDBu enhanced NCKX2 activity in a concentration-dependent manner, resulting in relative amplitudes of \( I_{\text{NCKX2}} \) at 100 nM, 500 nM, and 1 \( \mu \)M PDBu of 148 ± 9.6\% (\( n = 9 \)), 195 ± 8.3\% (\( n = 5 \)), and 306 ± 41\% (\( n = 4 \)), respectively (8 min treatment of PDBu). In contrast, the inactive PDBu analogue, 4\alpha\)-phorbol 12,13-dibutyrate (1 \( \mu \)M) did not change NCKX2 activity (Fig. 2C, 95.1 ± 6.8\%, \( n = 6 \)), suggesting that the effect of PDBu is dependent on the activation of PKC.

We examined the effects of PDBu (100 nM) on outward NCKX currents (\( I_{\text{NCKX}} \)) recorded from NCKX2-, NCKX3-, and NCKX4-expressing cells (Fig. 3). The enhancement of NCKX2 activity by 100 nM PDBu was significantly higher than that of NCKX3 or NCKX4, whose activity was little affected by PDBu. The relative amplitude of \( I_{\text{NCKX}} \) was estimated at 147 ± 7.7\% (\( n = 5 \)), 103 ± 17.5\% (\( n = 5 \)), and 105 ± 11.7\% (\( n = 6 \)) for NCKX2, NCKX3, and NCKX4, respectively (10-min treatment of PDBu). This result indicates that the PDBu-induced enhancement (PIE) is specific for NCKX2, and provides a basis for an intriguing possibility that such functional differences in members of the NCKX family may underlie their distinctive regulation.

The Enhancement of NCKX2 Activity by PDBu Is Dependent on the Activation of PKC—To confirm that the effect of PDBu on \( I_{\text{NCKX2}} \) was due to activation of PKC, we tested whether PKC inhibitors could prevent activation. When PDBu was washed out, the enhanced \( I_{\text{NCKX2}} \) slowly decreased to the control level over 10 min (data not shown). In contrast, when a PKC inhibitor was applied subsequent to PDBu, the enhancement of \( I_{\text{NCKX2}} \) was rapidly abolished within 2 min (Fig. 4B for chelerythrine; Fig. 4C for GF109203X). Moreover, in the presence of the PKC inhibitor, reapplication of 100 nM PDBu no longer enhanced \( I_{\text{NCKX2}} \). The effects of PKC inhibitors are summarized in Fig. 4D. Although PDBu alone enhanced \( I_{\text{NCKX2}} \), 148 ± 7\% (\( n = 6 \)) of the control, the PIE was significantly inhibited by 1 \( \mu \)M chelerythrine (94.7 ± 2\%, \( n = 4 \), \( p < 0.05 \)), a nonspecific PKC inhibitor, or by 100 nM GF109203X (82 ± 17\%, \( n = 4 \), \( p < 0.05 \)), an inhibitor for classic and novel type PKC (25, 26). To confirm the involvement of PKC in PIE, we studied the effects of PDBu in HEK293 cells that overexpress one of dominant negative isoforms of PKC together with NCKX2. The relative amplitude of \( I_{\text{NCKX2}} \) was significantly reduced in the HEK293 cells expressing the dominant negative PKC\( \delta \), PKC\( \theta \), or PKC\( \eta \). Consistently, pretreatment of Gö6976 (200 nM), an inhibitor of classic PKC and protein kinase D (or PKC\( \mu \)), had no significant effect on PIE (150 ± 4\%, \( n = 6 \), Fig. 4D). These results suggest that PIE is primarily mediated by PKC\( \delta \).

It has been reported that PKC can activate adenylate cyclase, protein kinase G, and protein kinase D. To investigate whether PKA or protein kinase G downstream of PKC is involved in PIE, we tested whether forskolin (10 \( \mu \)M) or 8-BrcGMP (100 \( \mu \)M) can directly enhance \( I_{\text{NCKX2}} \). Neither of these activators enhanced \( I_{\text{NCKX2}} \), suggesting that neither PKA nor protein kinase G is involved in PIE (supplemental Fig. S1C).
Moreover, Go6976 (200 nM), a protein kinase D inhibitor, had no significant effect on PIE, suggesting that protein kinase D is not involved in PIE (Fig. 4D).

Effects of PDBu on Ca2+ Decay Rate at the Calyx of Held—We tried to test whether PDBu can enhance the NCKX activity endogenously expressed at the calyx of Held, a large glutamergic presynaptic terminal in the medial nucleus of the trapezoid body (Fig. 5A), where NCKX, NCX, and the plasma membrane Ca2+-ATPase account for ~40%, 25%, and 20% of the total calcium clearance mechanisms (11). Because calyx of Held expresses large Ca2+-activated K+ current, it is not practical to isolate NCKX current. Thus, we studied the effects of PDBu on the Ca2+-decay rate at the calyx of Held.

When [Ca2+]i was measured using whole cell patch techniques, treatment of 200 nM PDBu typically increased resting [Ca2+]i and slowed down the Ca2+-decay rate of calcium transients (CaT) evoked by a short depolarizing pulse. To prevent a possible artifact caused by dilution of cytosolic components necessary for normal signal transduction pathway during whole cell patch recordings, we made a whole cell patch on the calyx of Held during a brief time (90 s) with a pipette solution containing 200 μM fura-4F, and gently withdrew the patch pipette. After the withdrawal, we could get fluorescence intensity comparable to 50–100 μM of fura-4F. We evoked CaTs by stimulating afferent axon fibers using a stimulation electrode located at the midline of the brain stem. About 15 or 20 action potentials at 200 Hz were required for obtaining a CaT whose amplitude was in the range of 1–1.5 M.

To inhibit the activity of NCX and plasma membrane Ca2+-ATPase, we included 200 μM exchanger inhibitory peptide (XIP) and 50 μM carboxyeosin in the pipette solution, which blocks NCX and plasma membrane Ca2+-ATPase, respectively (11, 30). Because Ca2+ clearance is steeply dependent on the level of [Ca2+]i, excur- sion from the resting value, we compared two CaTs only when the difference in the peak Δ[Ca2+]i levels between the two CaTs is <100 nM. Mean values for peak Ca2+ levels before and after PDBu application were 1.44 ± 0.28 μM and 1.42 ± 0.64 μM (n = 5).

Each Ca2+ transients recorded under these conditions were fitted with a bi-exponential function: y(t) = [Ca2+]rest
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A

\[ + A_1 \exp(-t \tau_1^{-1}) + A_2 \exp(-t \tau_2^{-1}) \]

We regarded the time derivative of the bi-exponential fit at \( t = 0 \) normalized with the peak Ca\(^{2+}\) amplitude,

\[ \lambda_1 = \frac{dy}{dt}|_{t=0} = \frac{\Delta[Ca^{2+}]_{\text{peak}}}{(A_1 \cdot \tau_1^{-1} + A_2 \cdot \tau_2^{-1})/(A_1 + A_2)} \]  

(Eq. 1)

as a parameter that represents the activity of Ca\(^{2+}\) clearance at the peak \( \Delta[Ca^{2+}] \) level (see Ref. 11 for details). The \( \lambda_i \) value in control conditions with internal XIP and carboxy eosin was 7.99 \( \pm \) 0.04 s\(^{-1}\) (n = 5), which was significantly slower than the mean \( \lambda_i \) value measured without XIP and CE included (14.0 \( \pm \) 0.90 s\(^{-1}\), n = 10). In the presence of XIP and carboxy eosin in the cytosol, PDBu clearly accelerated the Ca\(^{2+}\)-decay rate of the Ca\(^{2+}\) transient (Fig. 5B), and accordingly increased the \( \lambda_i \) to 12.88 \( \pm \) 1.27 s\(^{-1}\) (n = 5, p < 0.02, paired t test, Fig. 5C).

The total calcium clearance rate \( (d[Ca^{2+}]_i/dt) \) was calculated from the time derivative of the decay phase of the Ca\(^{2+}\) transient and calcium binding ratios of endogenous Ca\(^{2+}\) buffers and Ca\(^{2+}\)-indicator dye and plotted as a function of \( \Delta[Ca^{2+}]_i \) (Fig. 5D). The difference in the total calcium decay rate between Ca\(^{2+}\) transients before and after PDBu treatment is more pronounced at higher \( \Delta[Ca^{2+}]_i \) levels, indicating the NCKX activity is enhanced by PDBu.

Direct Phosphorylation of NCKX2 in Transfected HEK293 Cells—The above results show that PKC is a potent activator of the rat brain NCKX2. Next, we investigated whether PIE of INCKX2 involves a phosphorylation process. When NCKX2-expression HEK293 cells were dialyzed intracellularly with pipette solution containing alkaline phosphatase (50 units/ml), neither 100 nM nor 1 \( \mu \)M PDBu induced any significant enhancement of \( \lambda_{\text{NCKX2}} \) (96 \( \pm \) 2% (n = 5) and 88 \( \pm \) 4% (n = 3), respectively, supplemental Fig. S2). No enhancement of the outward \( \lambda_{\text{NCKX2}} \) in the presence of excess intracellular alkaline phosphatase indicates that the PDBu effect requires a phosphorylation process.

Phosphorylation of the NCKX2 protein molecule was examined by \(^{32}\)Porthophosphate labeling of HEK293 cells expressing FLAG-tagged NCKX2, followed by various treatments to stimulate PKC. The upper panels of Fig. 6A demonstrate that the overall incorporation of \(^{32}\)P into cellular proteins, and the level of expressed NCKX2, was equivalent under all conditions and was unaltered by PKC activation. The representative experiment shown in the lower panels of Fig. 6A illustrates that NCKX2 immunoprecipitated from untreated cells contained a constitutive level of phosphorylation, whereas no corresponding band was observed in immunoprecipitates from vector-transfected cells (lower left panel). Treatment with 0.1 \( \mu \)M PMA, but not with the inactive analogue, 4\( \alpha \)PMA, resulted in a decreased level of NCKX2 phosphorylation (lower left panel), whereas the amount of NCKX2 present in the respective immunoprecipitates was similar (lower right panel). The presence of 1 \( \mu \)M ionomycin had little effect on the enhanced phosphorylation. The averaged data from four independent experiments (Fig. 6B) indicated a significant 2- to 3-fold stimulation of \(^{32}\)P incorporation when normalized to NCKX2 protein. These data suggest that the level of NCKX2 phosphorylation was enhanced upon specific activation of PKC, whereas the lack of any effect of ionomycin suggests that a Ca\(^{2+}\)-independent isoform of PKC was responsible.

To ensure that PMA was effective in stimulating NCKX2 activity under these conditions, reverse-mode (i.e. Ca\(^{2+}\) entry) operation of NCKX2 was assessed by photometry of transfected HEK293 cells grown on coverslips, loaded with the fluorescent Ca\(^{2+}\)-indicator, fura-2, and mounted in a perfusion chamber on a microscope stage. A representative experiment is shown in Fig. 6C, and averaged data from three independent experiments are shown in Fig. 6D. NCKX2 activity was induced by a perfusion switch from Na\(^{+}\)- to Li\(^{+}\)-containing buffer, which caused an increase in Ca\(^{2+}\) entry and a rise in the fura-2 ratio (vector-transfected cells do not display this change, data not shown). Note that the rate of rise of the fura-2 ratio, as well as the peak value, due to NCKX2 activity typically decreases monotonically with repeated perfusion switches (Fig. 6, A and B). Under these
conditions, a 15-min treatment with 0.1 μM PMA induced an increase of ~2-fold in the rate of NCKX2 operation not observed with the inactive analogue, 4α-PMA (Fig. 6D). These data indicate that PMA treatment resulted in similar changes in NCKX2 activity as was observed upon PDBu treatment.

An increase in 32P incorporation in response to PMA stimulation was also observed for all three NCKX2 double mutants, even those without significant PIE of INCKX2 (data not shown). Due to the complexity of the phosphorylation experiment and the inherent variation in incorporation, our data were not sufficient to determine statistically significant differences in the amount of phosphorylation between the wild-type and mutant NCKX2 molecules. It was thus unclear if the maximal level of phosphorylation observed in these mutants was less than that of the wild type.

Thr-476 Is Critically Involved in PIE—A scan of the rat NCKX2 amino acid sequence for PKC recognition motifs (31) revealed three potential sites, as illustrated in Fig. 7A. To identify which of these sites might be phosphorylated by PDBu treatment, we mutated each putative PKC phosphorylation acceptor serine or threonine to alanine, either individually or in combination. These mutations were introduced into the fNCKX2, and the PDBu response was analyzed in transiently transfected HEK293 cells expressing each mutant. To confirm that these mutations did not affect expression and basal function of NCKX2, we measured the outward NCKX current density from cells expressing wild-type (WT) or each mutant (supplemental Table S1). No statistically significant difference in the current density between WT and mutants indicated that alanine substitution at the putative PKC phosphorylation sites, either individually or in combination, had negligible effects on the conformation and expression of NCKX2.

For each mutant, we examined the effect of treatment with 100 nM PDBu on the relative amplitude of INCKX2. Fig. 7B shows the time courses of PDBu effects on the relative amplitude of INCKX2 (right), together with representative current traces (left) recorded from wild-type and three single-site mutants. None of the single-site mutants were significantly different from wild type in terms of the relative amplitude of INCKX2 measured at 8 min of PDBu superfusion (148 ± 9.6% for WT (n = 9); 152 ± 21% for T166A (n = 7); 125 ± 10% for T476A (n = 9); 157 ± 6.5% for S504A (n = 13)). Although the INCKX2 of the T476A mutant was initially enhanced by PDBu, PIE became smaller 8–10 min after PDBu application, and the amplitude of INCKX2 eventually returned to the control level following 14 min of treatment (Fig. 7B). The biphasic response observed in the T476A mutant suggests that phosphorylation targets (Thr-166 or Ser-504) other than Thr-476 might undergo dephosphorylation by endogenous protein phosphatases (PPs) during the late phase of PDBu treatment, subsequent to the initial PKC-dependent phosphorylation.

To test this hypothesis, we examined the effect of various PPs
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inhibitors on the decline in PIE of INCKX2 observed in the T476A mutant (INCKX2-T476A). PP inhibitors were bath-applied 8–10 min following PDBo application, when the decline of INCKX2-T476A first becomes apparent. There are two kinds of PPs: Ca2+-independent and Ca2+-dependent PPs. PP-1 and PP-2A constitute the former group, and PP-2B (or calcineurin) belongs to the latter. We found that okadaic acid (1 μM), a specific inhibitor of PP-1 and PP-2A, had no effect on the decline of INCKX2-T476A (Fig. 7C). On the other hand, the specific inhibitors of calcineurin, cyclosporin A (1 μM) or FK-506 (1 μM), clearly prevented the decline phase normally observed in the presence of PDBo alone (Fig. 7C). The relative amplitudes of INCKX2-T476A measured 6 min following application of 100 nM PDBo alone, PDBo plus okadaic acid (1 μM), PDBo plus cyclosporin A (1 μM), or PDBo plus FK-506 (1 μM) were 96 ± 14% (n = 4), 115 ± 18% (n = 4), 133 ± 6% (n = 7, p < 0.05), or 139 ± 5% (n = 4, p < 0.05), respectively (p values indicate the statistical significance in the difference between PDBo alone and PDBo plus PP inhibitor). Unlike the T476A mutant, wild-type and other single mutants (T166A or S504A) exhibited no significant change in amplitude of INCKX2 following treatment with calcineurin inhibitors (Fig. 8).

It is well known that activation of calcineurin is dependent on an increase in cytosolic [Ca2+] (half-maximal [Ca2+] = 600 nM (32)). Thus, it was surprising that calcineurin was activated in the above experiments, considering that the cells were dialyzed with pipette solution containing 10 mM BAPTA. To test if

[Ca2+] rose high enough to activate calcineurin in our experimental conditions, we measured INCKX2-T476A and [Ca2+]i simultaneously by adding the Ca2+-indicator dye, fura-2 (500 μM) to the same patch pipette solution used in the above experiments (containing 10 mM BAPTA). Indeed, outward INCKX2 was accompanied by a Ca2+-transient whose peak [Ca2+]i level increased higher than 2.5 μM (supplemental Fig. S3A). Moreover, when INCKX2-T476A was induced every 4 min instead of every 2 min, the decline in PIE was not observed over a similar time course (supplemental Fig. S3B). These results indicate that the increase in cytosolic [Ca2+]i induced by reverse-mode INCKX2 under our experimental conditions was sufficient to activate calcineurin, which in turn dephosphorylated the T476A mutant, but not the other molecules. Thus, blocking the

FIGURE 5. Effects of PDBo on Ca2+ transients at the calyx of Held. A, a fluorescence image of the calyx of Held loaded with fura-4F. B, Ca2+ transients before (solid line) and after PDBo treatment (gray lines). Ca2+ transients were evoked by stimulation of afferent fibers of the calyx of Held (20 stimuli, 200 Hz), which were previously loaded with 200 μM fura-4F plus 200 μM exchanger inhibitory peptide (XIP) plus 50 μM carb oxemycin by a whole cell patch mode during a brief period (90 s). C, mean values for Ca2+-decay rate constant at the peak (λ2) before and after 200 μM PDBo treatment. Plots of λ2 for individual cases are superimposed. Error bars, ± S.E., p < 0.05 (paired t test). D, total calcium decay rate (d[Ca2+]i/dt) as a function of Δ[Ca2+]i. Values for d[Ca2+]i/dt were obtained from the time derivative (d[Ca2+]i/dt) of decay phases of the Ca2+ transients in A and calcium binding ratios of endogenous (λκ) and exogenous buffers (λκ) using d[Ca2+]i/dt = d[Ca2+]i/dt(λκ + λκ + 1). Each set of d[Ca2+]i/dt values were fitted with a fourth order polynomial function.

FIGURE 6. PKC stimulation induces phosphorylation of NCKX2. A, a typical experiment is illustrated in which vector- or FLAG-tagged NCKX2-transfected HEK 293 cells were labeled with 32P orthophosphate and treated with or without 1 μM ionomycin (Iono) and/or 0.1 μM PMA or 4a-PMA as indicated. Either 25 μg of protein extract from lysed cells (lysate) or FLAG-immunoprecipitated NCKX2 (FLAG-IP) were resolved on 9% SDS-PAGE gels and transferred to nitrocellulose membranes. The 32P autoradiograph (left panels) was recorded followed by immunoblotting with the anti-NCKX2 polyclonal antibody (right panels). B, the specific phosphate incorporation into the immunoprecipitated samples was determined by dividing the intensities of the bands on the 32P autoradiographs by those of the corresponding bands on the immunoblots. The mean values (± S.E.) from four separate experiments are shown. Statistically significant differences from the untreated sample are indicated with p < 0.01 (*) or p < 0.05 (**) using a paired ratio comparison t test. C, HEK293 cells grown on poly-d-lysine-coated coverslips were transiently transfected with FLAG-tagged NCKX2, loaded with 5 μM fura-2 AM, and mounted in a perfusion chamber on a microscope stage. The cells were perfused alternatively with either Na+ or Li+ buffer containing 5 mM K+. The fura-2 ratio from excitation at 340 nm and 380 nm was captured for the entire field of cells using a photometer. The perfusion was temporarily stopped, and the cells were incubated for 15 min with 0.1 μM PMA or 4a-PMA at the time indicated by the arrow. The reduction in both amplitude and rate of the rise in [Ca2+]i upon repetitive Li+ perfusion switches is typical also of untreated cells under these conditions. D, the mean initial rates (± S.E.) of increase in the fura-2 ratio for three pulses of NCKX2 activity were compared for PMA- or 4a-PMA-treated cells, respectively. Data were obtained from three separate experiments. *** statistically different from control (PMA pulse 1) with p < 0.01 using a paired ratio comparison t test, and from 4a-PMA pulse 2 with p < 0.05 using a t test to compare the rate data.
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In the present study, we have investigated the effect of PDBu on neuronal NCKX2 expressed in HEK293 cells, and shown evidence for the stimulation of NCKX2 activity by PKC, which may be mediated via direct phosphorylation of the exchanger. Recently, β-phorbol esters have been shown to activate not only decline in PIE by inhibiting calcineurin revealed that no single-site mutation at the three putative PKC phosphorylation sites completely inhibited the stimulation of INCKX2 induced by PDBu.

We hypothesized that multiple PKC consensus sites might be involved in the PKC-dependent stimulation of NCKX2 (33, 34). To test this hypothesis, similar studies were carried out with three double mutants: T166A/T476A, T476A/S504A, and T166A/S504A. Fig. 8A shows representative current traces for each mutant before and following 6 min of treatment with 100 nM PDBu (I-V curves recorded for WT and T476A/S504A are shown in supplemental Fig. S4). The two double mutants involving Thr-476 showed significantly reduced PIE of INCKX2.

The relative amplitudes of INCKX2 6 min after application of PDBu were 137 ± 10% for WT (n = 12), 99 ± 4.4% for T166A/T476A (n = 7, p < 0.05) and 104 ± 8% for T476A/S504A (n = 7, p < 0.05). On the other hand, the PIE of the T166A/S504A mutant was only slightly inhibited (131 ± 7%, n = 10). Finally, to rule out the possibility that activation of calcineurin might be responsible for the loss of PKC effects on the double mutants, we examined effects of 1 μM FK-506 on PIE of INCKX2 from the cells expressing each double mutant. When FK-506 was added 8 min following PDBu application, no significant effect on INCKX2 was observed for any of the double mutants (Fig. 8B).

The relative amplitudes of INCKX2 in the presence of PDBu plus FK506 were 157 ± 8% for WT (n = 8), 105.6 ± 6.6% for T166A/T476A (n = 7), 102 ± 10% for T476A/S504A (n = 7) and 134 ± 14% for T166A/S504A (n = 6). This lack of FK-506 effect indicates that activation of calcineurin was not related to the loss of PIE in the double mutants.

The above results suggest that multiple PKC consensus sites are involved in PIE of NCKX2. To evaluate the contribution of each PKC site to PIE, the mean values for relative amplitude of INCKX2 of wild-type and various mutants measured at 14 min after PDBu treatment in the presence of 1 μM FK506 were sorted and plotted on the bar graph in Fig. 9. The PIE was higher in the T166A/S504A (Thr-476 is the available target for PKC) mutant than in the T476A/S504A (Thr-166 is available) or T166A/T476A (Ser-504 is available) mutant, suggesting that these sites are not functionally equivalent, but each contributes to PIE with a different weighting factor (w). In addition, from the observation that PIE was higher in the T476A mutant (both of Thr-166 and Ser-504 are available) than T166A/T476A or T476A/S504A (either Thr-166 or Ser-504 is available), it could be inferred that either Thr-166 or Ser-504 is not sufficient to yield PIE, but both sites (Thr-166 and Ser-504) contribute cooperatively to induce PIE.

DISCUSSION

In the present study, we have investigated the effect of PDBu on neuronal NCKX2 expressed in HEK293 cells, and shown evidence for the stimulation of NCKX2 activity by PKC, which may be mediated via direct phosphorylation of the exchanger. Recently, β-phorbol esters have been shown to activate not only

The values for the amplitude of INCKX2(T476A) were normalized to their corresponding control amplitudes. Error bars represent ± S.E. Asterisks denote statistical significance between PDBu alone and PDBu plus cyclosporin A or PDBu plus FK-506 (*, p < 0.05).
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FIGURE 8. Effects of double-site mutations on PIE of outward \( \Delta \text{INCKX2} \). A, representative recordings of NCKX2 current \( \Delta \text{INCKX2} \) recorded from HEK293 cells expressing the mutants T166A/T476A (upper), T476A/S504A (middle), or T166A/S504A-\( \Delta \text{NCKX2} \) (lower). Each trace of \( \Delta \text{INCKX2} \) in control condition (gray) is superimposed on the corresponding one in the presence of 100 nM PDBu (black). B, time courses for the change in normalized amplitude of \( \Delta \text{INCKX2} \) of WT and double mutants before and during the application of 100 nM PDBu alone or with 1 \( \mu \)M FK506: WT (circle), T166A/T476A (n = 8, △), T476A/S504A (n = 6, ○), and T166A/S504A (n = 6, ▽). INCKX2 was elicited every 2 min by applying \( \Delta \text{K}^+ \) solution for 15–20 s. In all the experiments, amplitude of \( \Delta \text{INCKX2} \) was initially monitored for 4 min and then for 8 min in the presence of 100 nM PDBu alone (horizontal bar) and for 8 min in the presence of 100 nM PDBu plus 1 \( \mu \)M FK506 (horizontal line). Values for the amplitude of \( \Delta \text{INCKX2} \) in each experiment were normalized to that in control condition. Error bars: ± S.E.

FIGURE 9. Mean values for relative \( \Delta \text{INCKX2} \) of wild-type and various mutants measured at 14 min following PDBu treatment in the presence of 1 \( \mu \)M FK506. The relative contribution \( (w) \) of each putative PKC phosphorylation site was calculated from double mutants in which only a single site relevant to PIE is assumed to be available for PKC. The \( w \) values calculated in this way for Thr-166, Thr-476, and Ser-504 were 0.05, 0.6, and 0.05, respectively. The expected value for relative \( \Delta \text{INCKX2} \) in each mutant was calculated from simple addition of \( w \) values of available phosphorylation sites in each mutant (inverted triangles). To correct the discrepancy between the real and expected values, different cooperativity factors \( (\omega) \) were multiplied to the summed \( w \) values, and the resultant expected value for each mutant is shown by triangles. The \( \omega \) values between Thr-166 and Ser-504 and between Thr-476 and other sites were assumed to be 6.7 and 1.5, respectively. Error bars: ± S.E., n = number of cells studied.

PKC but also Munc13, which enhances fusion of secretory vesicles to the plasma membrane (35). We think it unlikely that the PIE of NCKX2 function we have observed is caused by increased vesicular insertion to the plasma membrane by Munc13 (36) for the following reasons. 1) PIE was not observed in other NCKX isoforms (NCKX3 or NCKX4), as might be expected if PIE were mediated via a general vesicle insertion mechanism; 2) PIE of the NCKX2 current was blocked by PKC inhibitors GF109203X or chelerythrine or by inclusion of alkaline phosphatase in the recording pipette; 3) NCKX2 double mutants at PKC phosphorylation motifs (T166A/T476A and T476A/S504A) abolished the PIE, suggesting a direct activation of NCKX2 by PKC; and 4) enhanced phosphorylation of the exchanger protein itself was observed in the presence of PMA. Thus, it appears that NCKX family members expressed in the brain are differentially regulated by PKC. To our knowledge, this is the first report on the modulation of an NCKX family member via protein kinases, although there are many studies on such regulation in the NCX family.

**PKC Isoform Involved in PIE and Downstream Mechanisms**—There are three categories of PKC isoforms according to the dependence of their activation on calcium and diacylglycerol. In the present study, two or three episodes of \( \Delta \text{INCKX2} \) were induced prior to the application of PDBu. During this period, \( \left[ \text{Ca}^{2+} \right] \) varied typically in the range of 10 to 150 nM, because the cells were intracellularly perfused with 10 mM BAPTA (supplemental Fig. S3). The \( \left[ \text{Ca}^{2+} \right] \) level prior to the induction of the very first enhanced \( \Delta \text{INCKX2} \) episode in the presence of PDBu was well below 100 nM, suggesting that \( \text{Ca}^{2+} \)-independent novel PKCs might be involved in the PIE. Consistent with this observation, PMA enhanced the \( \text{Ca}^{2+} \) incorporation into NCKX2 without pre-treatment of ionomycin. Moreover, we showed that overexpression of the dominant negative mutant of PKC\( \varepsilon \) significantly reduced PIE. Interestingly, it was reported that a novel type PKC (PKC\( \varepsilon \)) but not classic PKC is expressed at the presynaptic terminal, calyx of Held, where NCKX activity was demonstrated (11, 37).

The present study suggests that PIE may involve multiple PKC sites on the molecule NCKX2. Previous studies on PKC-dependent modulation of ionic channels indicate that PKC modulates channel activity not only by the modulation of the intrinsic channel properties but also by the regulation of receptor/channel trafficking. Modulation of intrinsic channel properties by PKC involves usually direct phosphorylation of the channel protein (38, 39). On the other hand, PKC-dependent regulation of channel trafficking involves diverse mechanisms. PKC promotes insertion of N-methyl-D-aspartate channels or causes internalization of ATP-sensitive \( \text{K}^+ \) channel through phosphorylation of channel-associated protein(s) involved in signaling and/or trafficking without phosphorylation of the channel protein itself (40, 41). However, PKC-induced internalization of GluR2 and surface expression of Kir1.1 require phosphorylation of the channel itself (42, 43). Although the inhibition of the PIE by point mutation favors the former scenario (change in intrinsic properties of the NCKX2 transporter), because the mechanism that leads
from phosphorylation of NCKX2 to enhanced activity, further experiments will be required to distinguish unambiguously between recruitment of new transport units to the plasma membrane or the enhancement in intrinsic activity of existing units.

**Does Direct Phosphorylation of NCKX2 Induce PIE?**—To address this issue, we carried out phosphorylation experiments similar to those of Fig. 6 with the three double mutants. An increase in $^{32}$P incorporation in response to PMA was observed for all double mutants, even those that exhibited negligible PIE of INCKX2 (data not shown). However, it was unclear if the maximal level of phosphorylation observed in these mutants was significantly less than that of the wild type. We believe two possible explanations may account for the discrepancy between the functional effects and direct phosphorylation of the NCKX2 mutants. First, NCKX2 may be phosphorylated in a PKC-dependent manner at sites in addition to Thr-166, Thr-476, and Ser-504. Although these other sites do not affect activity directly, the background incorporation prevents observation of only those phosphorylation events directly linked to activity changes. The activity-independent phosphorylation events may occur at PKC sites that do not match the typical consensus motif, or may be indirect, due to PKC activation of a kinase with different specificity. Second, it is possible that the mutations we have introduced do not alter PKC-dependent phosphorylation directly but, rather, have the effect of uncoupling phosphorylation from the increase in activity via a change in protein structure. Even if phosphorylation of Thr-476 is not a direct cause of PIE, our point-mutation studies suggest that Thr-476 plays a critically important role. Moreover, among the mutants that exhibited significant PIE, only the T476A mutant was susceptible to calcineurin, indicating that Thr-476 is necessary not only for the induction of PIE but also for maintenance of PIE by protecting other susceptible phosphorylated sites present on NCKX2 from subsequent dephosphorylation by calcineurin.

The PKC consensus sequences involving Thr-166, Thr-476 or Ser-504 found in NCKX2 are not conserved in NCKX3 or NCKX4. All three of these NCKX family members possess putative PKC-phosphorylation sites in regions of the proteins that are likely to be cytoplasmically exposed. The fact that only NCKX2 was overtly affected by PD8a indicates that only a subset of PKC consensus sites are available in the native proteins or that functional changes in exchanger properties result only from phosphorylation of specific sites.

In summary, the present study demonstrates that multiple amino acid residues are involved in PKC-mediated stimulation of NCKX2, with Thr-476 in the large intracellular loop serving as the critical residue for mediating enhancement of exchanger activity. In contrast, the activity of NCKX3 and NCKX4 was not regulated by a pathway involving PKC. This regulatory difference between family members, which is also found in other transporters, could provide an important physiological mechanism by which the multiplicity of protein functions can be generated.

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