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Full-length cardiac Na^+/Ca^{2+} exchanger 1 protein is not phosphorylated by protein kinase A

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Wanichawan P, Louch WE, Hortemo KH, Austbø B, Lunde PK, Scott JD, Sejersted OM, Carlson CR. Full-length cardiac Na^+/Ca^{2+} exchanger 1 protein is not phosphorylated by protein kinase A. *Am J Physiol Cell Physiol* 300: C989–C997, 2011. First published February 2, 2011; doi:10.1152/ajpcell.00196.2010.—The cardiac Na^+/Ca^{2+} exchanger 1 (NCX1) is an important regulator of intracellular Ca^{2+} homeostasis and cardiac function. Several studies have indicated that NCX1 is phosphorylated by the cAMP-dependent protein kinase A (PKA) in vitro, which increases its activity. However, this finding is controversial and no phosphorylation site has so far been identified. Using bioinformatic analysis and peptide arrays, we screened NCX1 for putative PKA phosphorylation sites. Although several NCX1 synthetic peptides were phosphorylated by PKA in vitro, only one PKA site (threonine 731) was identified after mutational analysis. To further examine whether NCX1 protein could be PKA phosphorylated, wild-type and alanine-substituted NCX1-green fluorescent protein (GFP)-fusion proteins expressed in human embryonic kidney (HEK)293 cells were generated. No phosphorylation of full-length or calpain- or caspase-3 digested NCX1-GFP was observed with purified PKA-C and [γ-^{32}P]ATP. Immunoblotting experiments with anti-PKA substrate and phosphothreonine-specific antibodies were further performed to investigate phosphorylation of endogenous NCX1. Phospho-NCX1 levels were also not increased after forskolin or isoproterenol treatment in vivo, in isolated neonatal cardiomyocytes, or in total heart homogenate. These data indicate that the novel in vitro PKA phosphorylation site is inaccessible in full-length as well as in calpain- or caspase-3 digested NCX1 protein, suggesting that NCX1 is not a direct target for PKA phosphorylation.

calpain; caspase; human embryonic kidney 293; peptide array

THE CARDIAC Na^+/Ca^{2+} exchanger 1 (NCX1) is an important regulator of intracellular Ca^{2+} homeostasis and plays an essential role in the excitation-contraction-relaxation cycle. The NCX1 protein is composed of ~970 amino acids. As a mature protein, NCX1 comprises nine transmembrane segments (TMs), two pore-forming regions between TM2 and TM3 and between TM7 and TM8, and a large hydrophilic loop of ~550 amino acids between TM5 and TM6 (28, 38). NCX1 transports 1 Ca^{2+} ion across the membrane in exchange for 3 Na^+ ions. Although its primary function is to extrude Ca^{2+} from the cytosol during action potential (AP) repolarization (forward mode), NCX1 can also facilitate Ca^{2+} entry under certain conditions (reverse mode). The extent of Ca^{2+} extrusion by NCX1 varies between species (2). In human cardiomyocytes, NCX1 contributes to almost 30% of the total Ca^{2+} removal from the cytosol during the declining phase of the Ca^{2+} transient (13) and is thus essential to maintaining low intracellular [Ca^{2+}] during diastole. Numerous studies have reported significantly increased NCX1 expression and/or activity in development of cardiac hypertrophy and heart failure in animal models and humans (6, 10, 11, 14, 29, 30, 43, 44). However, it remains unclear whether such alterations are beneficial by compensating for decreased Ca^{2+}-ATPase (SERCA) activity or deleterious by contributing to arrhythmia (32).

NCX function is regulated by a variety of extracellular and intracellular cations, signaling molecules, hormones, and peptides (39). Although several studies indicate that β-adrenergic receptor activation (β-AR) modulates NCX, there are conflicting reports as to whether PKA exerts effects on NCX1 directly (9, 20, 44). Several studies indicate that NCX1 is phosphorylated by PKA in vitro (16, 33, 44) and that the phosphorylation sites are located in the large intracellular loop (16). However, no phosphorylation site has so far been identified, and it remains unclear whether NCX1 is in fact a physiological substrate of PKA.

In this study, we screened NCX1 for PKA phosphorylation sites by bioinformatic analysis and extensive use of peptide arrays. By mutational analysis, we identified a putative PKA phosphorylation site at threonine 731 in the large intracellular loop. However, we observed that full-length as well as calpain- and caspase-3 digested NCX1-green fluorescent protein (GFP) were not phosphorylated with purified PKA-C and [γ-^{32}P]ATP. Furthermore, endogenous NCX1 phosphorylation levels were not increased in neonatal cardiomyocytes, LV tissue lysates, or rats treated with forskolin or isoproterenol. Thus, although a putative PKA phosphorylation site was identified in vitro, our data indicate that full-length and calpain- or caspase-3-digested NCX1 are not direct physiological substrates of PKA.

MATERIALS AND METHODS

The investigation conforms to the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996) and was approved by the Norwegian National Animal Research Committee.

Peptide synthesis. The intracellular loop (amino acids 243–800) of rat NCX1 protein (EDM02743) was synthesized as 20-mer peptides with three or five amino acid offsets on cellulose membranes using a Multipep automated peptide synthesizer (INTAVIS Bioanalytical Instruments, Koeln, Germany) as described (8). Phospho-NCX1 (pThr731-NCX1) peptide (CFKSTVDLIKKpTNL) and plain NCX1 peptide (CFKSTVDLIKKTNL) were synthesized and purified to >90% purity using high-performance liquid chromatography (Genscript).

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In vitro phosphorylation. Peptides synthesized on membranes were subjected to phosphorylation according to Carnegie et al. (4). Briefly, the membranes were activated in methanol, washed five times in TBST, and then incubated in a blocking solution [20 mM HEPES, (pH 7.4) 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mg/ml BSA, and 100 μM MgATP] at 4°C overnight. The membranes were then submerged into membrane phosphorylation (MP) buffer containing 20 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.2 mg/ml BSA, 50 μM MgATP, 0.1 μg catalytic subunit PKA (Upstate Biotechnology, Lake Placid, NY), and 10 μCi [γ-^{32}P]ATP (Montebello, Oslo, Norway). In vitro phosphorylation was performed at 30°C for 30 min with agitation. The membranes were washed and dried as described previously (12). The peptide sequence MPGTIEVKVL RRASNIGIVY containing the consensus PKA phosphorylation site RRxS was used as positive control. NCX1 immunocomplexes were washed in kinase buffer [25 mM Tris (pH 7.5), 0.1 mM EGTA, 0.1 mM Na_3VO_4, 0.03% Brij-35, 10 mM $MgCl_2$, and 0.1 mM ATP] and resuspended in 50 μl kinase buffer containing 2.5 μCi [γ-^{32}P]ATP (Montebello) and 0.1 μg recombinant PKA-C (Upstate Biotechnology). The PKA inhibitor PKI (10 μM, Calbiochem, San Diego, CA) was used as a control for specificity. The phosphorylation reaction was carried out at 30°C for 30 min and terminated by adding 4× loading buffer before SDS-PAGE analysis. The membranes were exposed to Fujifilm phosphor imaging plates. Autoradiography was performed using a BAS-1800 phosphor imager (Fuji Medical Systems).

NCX1-GFP constructs. MGC clone BC079673 was cloned into pEGFP-N1 using *Hind*III and *BamH*I (Genscript, Piscataway, NJ). Alanine mutations (S722A, T723A, and T731A) in NCX1-GFP were also performed by Genscript. The fidelity of the cloning procedure and mutagenesis were verified by sequence analysis.

Cell culture and transfection of HEK293 cells. Human embryonic kidney (HEK)293 cells were cultured in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 1% nonessential amino acids and maintained in a 37°C, 5% CO_2 humidified incubator. The HEK293 cells were transfected with NCX1-GFP contructs using Lipofectamine 2000 as instructed by the manufacturer (Invitrogen Dynal, Oslo, Norway). After 24 h, the cells were lysed in lysis buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100 with complete protease inhibitor cocktail tablets (Roche Diagnostics, Oslo, Norway).

Isolation and stimulation of neonatal rat cardiomyocytes. The procedure for isolation of neonatal rat cardiomyocytes was conducted as described previously (7). Cells were stimulated with 1 μM isoproterenol (The Norwegian Pharmacy Association, Oslo, Norway) or 10 μM forskolin (Sigma, St. Louis, MO) for 5 or 10 min, respectively, before being harvested in IP buffer [20 mM HEPES, (pH 7.5) 150 mM NaCl, 1 mM EDTA, and 1% Triton].

Isoproterenol infusion of rats. Rats (320 ± 2 g) were anesthetized with 2% isoflurane in 25%:75% O_2-N_2O, intubated, and placed on a ventilator (Zoovent Triumph Technical Services, London, UK). Aortic blood pressure was monitored by a pressure-sensitive catheter (Millar instruments, Houston, TX) inserted through the left carotid artery. Isoproterenol (isoprenaline 0.2 mg/ml, The Norwegian Pharmacy Association) (0.25 ± 0.03 ml) was infused through a venflon in the left jugular vein until the systolic blood pressure decreased to 60 ± 2% of the pressure before infusion (133 ± 6 mmHg). Control animals were infused with 0.3 ml 0.9% NaCl. After 5 min, the heart was excised, and the left and right ventricles were separated and frozen in liquid nitrogen.

Isolation of left ventricle lysates. Frozen left ventricles from rats were pulverized in a mortar with liquid nitrogen before transfer to lysis buffer [20 mM HEPES, (pH 7.5) 150 mM NaCl, 1 mM EDTA, and 0.5% Triton] with protease (1 mM PMSF and Complete Mini EDTA-free tablets; Roche Diagnostics) and phosphatase inhibitors (1 mM Na_3VO_4 and 50 mM NaF) in a glass tube. The samples were homogenized three times for 1 min at 0°C with a Polytron 1200 and centrifuged at 70 000 *g* for 60 min at 4°C. Supernatants were collected and stored at −70°C before determination of the protein concentration and further analyses. Protein concentrations were determined by Micro BCA Protein Assay Kit (Pierce, Rockford, IL).

PKA activation of LV lysates. LV lysates were incubated with 10 μM forskolin, 250 μM IBMX (Sigma-Aldrich, Oslo, Norway), phosphatase inhibitors 1 μg/ml cyclosporin A (Sigma-Aldrich) and 50 μM NaF (Sigma-Aldrich) for 20 min at 30°C before immunoprecipitation and Western blot analysis.

Antibodies. Immunoblotting and immunoprecipitation were carried out using anti-NCX1 (custom made, Genscript), anti-pThr (no. 9381, Cell Signaling Technology, Beverly, MA), anti-PKA substrate (no. 9621, Cell Signaling), anti-pThr731-NCX1 (custom made, Genscript), anti-pSer16-PLB (A010-12, Badrilla, Leeds, UK), anti-GAPDH (V-18, SC-20357, Santa Cruz Biotechnology), and anti-GFP (polyclonal, Clontech, Mountain View, CA). Horseradish peroxidase-conjugated anti-mouse and donkey anti-rabbit IgG (both from GE HealthCare) were used as secondary antibodies.

Immunoprecipitation. NCX1 was immunoprecipitated from HEK293 cell lysates, LV lysates, or neonatal rat cardiomyocytes with a custom-made NCX1 antibody (Genscript) or a GFP polyclonal antibody (Clontech) using true blot anti-rabbit IgG IP beads (eBioscience, San Diego, CA) or protein A/G agarose beads (Santa Cruz Biotechnology) overnight at 4°C. Immunocomplexes were washed three times in lysis buffer before being boiled in SDS loading buffer and analysis by immunoblotting. Rabbit IgG (Santa Cruz Biotechnology) was used as a negative control.

Immunoblot analysis. Lysates and immunoprecipitates were analyzed on a 4%–15% or a 4%–20% SDS/PAGE, while 16.5% Tris-Tricine gels were used to resolve peptides. Samples were then transferred to PVDF membranes. The PVDF membranes and peptide arrays were blocked in 5% nonfat dry milk, 5% BSA, or 1% blocking solution (Western Blocking Reagent, Roche Diagnostics) in TBST for 60 min at room temperature, incubated overnight at 4°C with primary antibodies, washed five times 5 min in TBST, and incubated with a horseradish peroxidase-conjugated secondary antibody. Blots were developed by using ECL Plus (GE HealthCare, Oslo, Norway). The chemiluminescence signals were detected by Las 1000 or Las 4000 (Fujifilm, Tokyo, Japan).

Densitometric analysis. Densitometric analysis was performed using Image Gauge 3.46, Science lab 99 (Fujifilm), Image Quant (Fujifilm), or Scion Image (Scion, Frederick, MD).

In vitro cleavage of NCX. Immunoprecipitated NCX1 or LV lysates from adult rats were washed and resuspended (or diluted) in calpain buffer containing 10 mM EGTA, 0.1% Triton, 100 mM HEPES (pH 7.5), and 20 mM $CaCl_2$. Subsequently, 1 μg of μ-calpain (Calbiochem) was added to the suspension, and proteolysis was performed at 37°C for 30 min. For caspase-3 cleavage, caspase-3 buffer containing 50 mM Tris (pH 7.4), 1 mM EDTA, and 10 mM DTT were used. The reaction was performed at 37°C for 1 h. The cleavage reactions were stopped by adding 4× SDS loading buffer and analyzed by immunoblotting.

RESULTS

Bioinformatic analysis of NCX1. Human, rat, and mouse NCX1 protein sequences were screened in silico for consensus PKA sites using pkaPS (http://mendel.imp.ac.at) (24) and Scansite software (http://scansite.mit.edu). Six consensus PKA sites were identified in human NCX1, whereas seven consensus sites were identified in rat and mouse NCX1 (Table 1). Several of these sites were located in the large intracellular loop, which has been suggested to contain most of the interaction and regulation sites (Fig. 1). Four potential cytoplasmic PKA sites were identified in the mouse, rat, and human NCX1 [KAVS, VRVS (rat, mouse)/VKVS (human), LRTS, and KNKT],

Table 1. *PKA phosphorylation sites identified in NCX1 in silico*

Position	Sequence	Region*	Reference
		NCX1 human (P32418)	
8	RRL**S**	Precursor	Motif scan
79	ARA**T**	TM1	Motif scan
392	KAV**S**	Cytoplasmic (between TM5 and TM6)	pkaPS
501	VKV**S**	Cytoplasmic (between TM5 and TM6)	pkaPS
564	LRT**S**	Cytoplasmic (between TM5 and TM6)	pkaPS
621	KNK**T**	Cytoplasmic (between TM5 and TM6)	Motif scan
		NCX1 rat (Q01728)	
18	RLV**T**	Precursor	Motif scan
76	ARA**T**	TM1	Motif scan
123	TKT**T**	Extracellular (between TM2 and TM3)	Motif scan
389	KAV**S**	Cytoplasmic (between TM5 and TM6)	pkaPS, motif scan
498	VRV**S**	Cytoplasmic (between TM5 and TM6)	pkaPS
561	LRT**S**	Cytoplasmic (between TM5 and TM6)	pkaPS
618	KNK**T**	Cytoplasmic (between TM5 and TM6)	Motif scan
		NCX1 mouse (A001590)	
76	ARA**T**	Transmembrane (M1)	Motif scan
123	TKT**T**	Extracellular (between TM2 and TM3)	Motif scan
389	KAV**S**	Cytoplasmic (between TM5 and TM6)	pkaPS, motif scan
498	VRV**S**	Cytoplasmic (between TM5 and TM6)	pkaPS
499	RVS**S**	Cytoplasmic (between TM5 and TM6)	pkaPS
561	LRT**S**	Cytoplasmic (between TM5 and TM6)	pkaPS
618	KNK**T**	Cytoplasmic (between TM5 and TM6)	Motif scan

PKA, protein kinase A; NCX1, Na^+/Ca^{2+} exchanger 1; TM, transmembrane segment. pkaPS: Only good hits (score ≥0), motif scan: low-high stringency. *See Ref. 30a. See text for more details.

whereas an additional site, RVSS, was only identified in the mouse (Table 1). The consensus site KAVS, identified by both pkaPS- and Scansite, has previously also been suggested to be a putative PKA phosphorylation site in canine and frog NCX1 (17, 25). Three consensus PKA sites RRLS/RLVT, ARAT, and TKTT, were respectively located in the precursor sequence, NH_2-terminus of TM1, and a small extracellular domain between TM2 and TM3. These could be excluded as potential PKA phosphorylation sites due to their position (Fig. 1, precursor sequence not shown). No consensus PKA sites were identified in the small cytoplasmic regions between TM1 and TM2, TM3 and TM4, or in the cytoplasmic COOH-terminus.

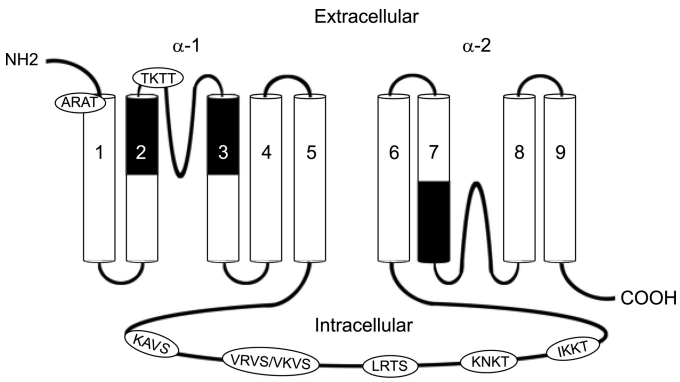

Fig. 1. Localization of putative protein kinase A (PKA) phosphorylation sites placed in the topological model of the cardiac Na^+/Ca^{2+} exchanger 1 (NCX1) (28). The nine transmembrane segments are indicated with open areas, and the two β-repeats are shown in filled areas. In silico screening identified 4 consensus PKA sites located between transmembrane *segment 5* and *6* (the large intracellular loop) of NCX1: KAVS, VRVS (mouse, rat)/VKVS (human), LRTS, and KNKT. An additional consensus site was identified in the intracellular loop in mouse NCX1 (RVSS, not shown). In vitro PKA phosphorylation identified phosphorylation site IKKT, which was conserved between the 3 species. In addition, ARAT was identified in the NH_2-terminus of TM1, and TKTT (only mouse and rat) was identified between transmembrane *segments 2* and *3*.

PKA phosphorylation of NCX1 synthetic peptides. The large intracellular loop of rat NCX1 that contains four consensus PKA sites was screened for phosphorylation in vitro. An immobilized array with overlapping 20-mer synthetic peptides covering the large intracellular loop was subjected to PKA phosphorylation. Peptides containing the consensus site LRTS were modestly phosphorylated, but very little phosphorylation was observed for peptides containing consensus sites KAVS, VRVS, or KNKT (Fig. 2*A*, *left*, underlined). In addition, phosphorylation was observed for peptides containing nonconsensus PKA sites: QVLS, QQKS, RLMT, GGFT, FTLT, EFKS, FKST, IKKT, and HFLT (Fig. 2*A*, not underlined). The most robust phosphorylation was observed for the peptide sequence SYEFKSTVDKLIKKTNLALV, which contains three putative phospho-acceptor sites: Ser722, Thr723, and Thr731. As positive control, a peptide sequence containing the consensus PKA site RRAS was used (Fig. 2*A*, *right*).

γ-^{32}P-labeled positive peptides identified in Fig. 2*A* were substituted for the nonphosphorylatable side chain alanine and incubated with PKA. As shown in Fig. 2*B*, mutation to alanine had little effect on the phosphorylation of most of the peptides. However, mutation T731A in the context of an IKKT motif abolished PKA phosphorylation (Fig. 2*B*). This identified threonine 731 as a target for PKA phosphorylation in vitro. Notably, strong phosphorylation was observed for single mutated peptides S722A or T723A, and reduced phosphorylation was detected when the peptide was double mutated (S722A, T723A) (Fig. 2*C*). This suggests that Ser722 and Thr723 might contribute to the phosphorylation level of the peptide.

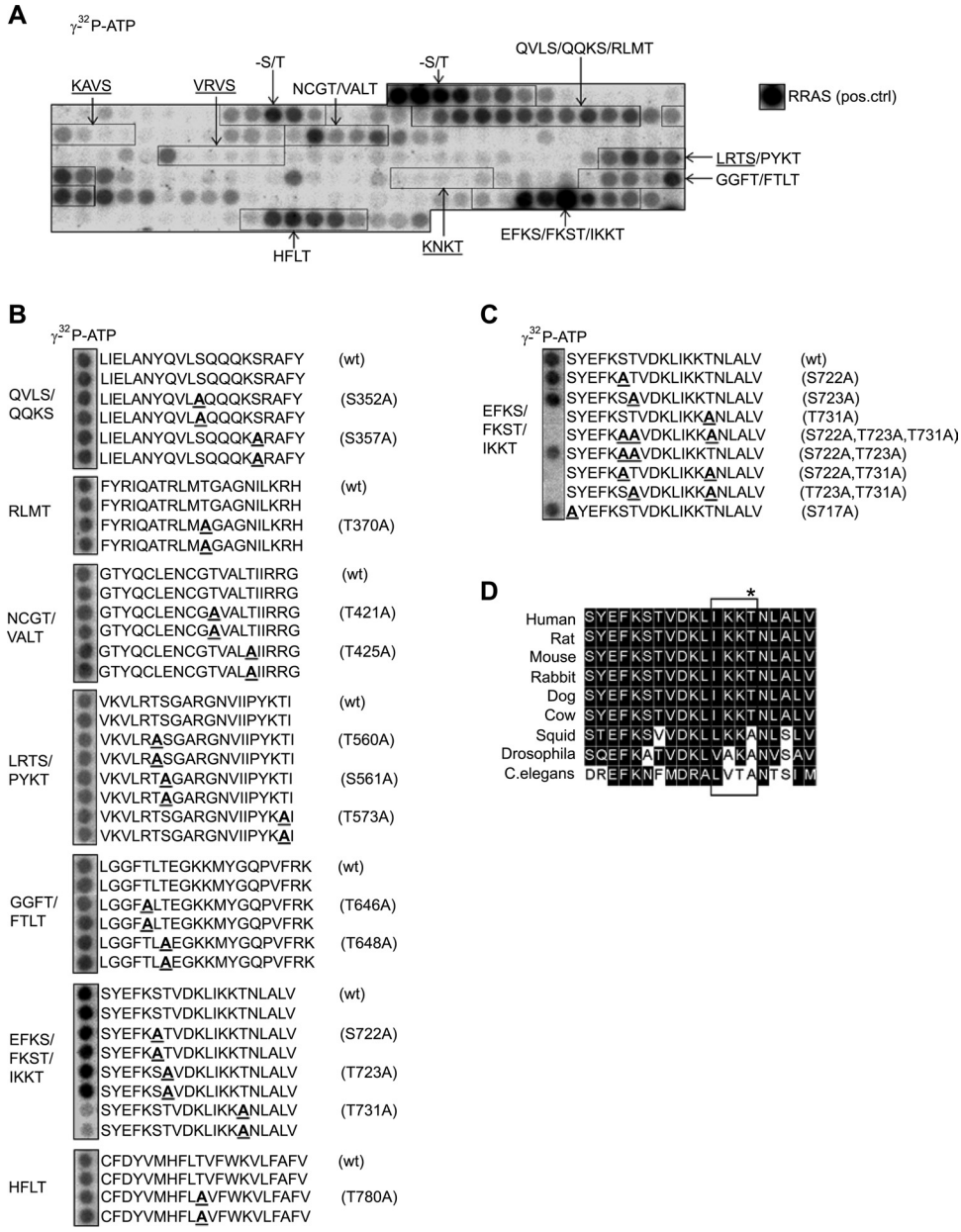

Fig. 2. PKA phosphorylation of NCX1 in vitro. *A*: mapping of PKA phosphorylation sites in NCX1. Incorporation of [γ-^{32}P]phosphate into 20-mer peptides with 3 amino acids offset encompassing the intracellular loop of rat NCX1 (amino acids 243–800). Outlined boxes denote putative PKA phosphorylation sites. Underlined amino acids were identified as consensus PKA sites by bioinformatic analysis. Some positive peptide sequences did not contain a putative phospho-acceptor serine or threonine (−S/T). A peptide containing the consensus PKA site RRAS was used as positive control (at *right*). Single mutation analysis (*B*) and double and triple mutation analysis (*C*) of putative phosphorylation sites are shown as identified in *A*. One putative PKA phosphorylation site was confirmed by replacing the phospho-acceptor serine or threonine with alanine. Incorporation of [γ-^{32}P]phosphate was detected by BAS in all experiments. *D*: alignment of the particular NCX1 peptide sequence from different species (DNA Star, Madison, WI). Filled black boxes indicate similar amino acids within the consensus, and the bracket at top indicates the putative PKA phosphorylation site. *Putative phospho-acceptor threonine in mammalian NCX1 (Thr731).

Alignment of NCX1 from different species showed that the in vitro PKA phosphorylation site IKKT was conserved in the human, rat, mouse, rabbit, dog, and cow but was not present in lower organizms such as squid, *Drosophila*, or *Caenorhabditis elegans* (Fig. 2*D*, boxed area). Interestingly, squid, *Drosophila*, and *C. elegans* contain an alanine at the corresponding site (denoted with an asterisk).

No PKA phosphorylation of NCX1-GFP. To further analyze whether the intact NCX1 could be PKA phosphorylated, wild-type and alanine-substituted NCX1-GFP forms (S722A, T723A, and T731A) were transfected into HEK293 cells. The fusion proteins were tested for their ability to be phosphorylated (constructs schematic illustrated in Fig. 3*A*). The NCX-GFP fusion proteins were immunoprecipitated with polyclonal GFP antibodies and phosphorylated with purified PKA-C and [γ-^{32}P]ATP. To secure consistent PKA phosphorylation, serine/threonine phosphatase inhibitors (NaF and cyclosporin A) were also included in the assay. Very few γ-^{32}P-labeled positive proteins were observed when NCX-GFP was subjected to immunoprecipitation (Fig. 3*B*, *top*). A very weak 150-kDa γ-^{32}P-labeled positive protein was observed for wild-type (*lane 1*) and alanine-substituted NCX-GFP proteins (*lanes 3*, *4*, and *5*) but also in the presence of the PKA inhibitor PKI (*lane 2*), suggesting the phosphorylation to be nonspecific. As shown in Fig. 3*B*, *bottom*, equal amounts of the NCX-GFP fusion proteins were precipitated. Phosphorylation of PKA-RI (A98S) mutated to contain an autophosphorylation site (3) was used as positive control (data not shown).

No phosphorylation of endogenous NCX following PKA activation. Several experiments were performed to investigate whether endogenous NCX1 could be phosphorylated following PKA activation. First, a custom-made antibody highly specific to an NCX1-specific region was generated (Fig. 4*A*, underlined sequence). Importantly, the antibody epitope did not overlap

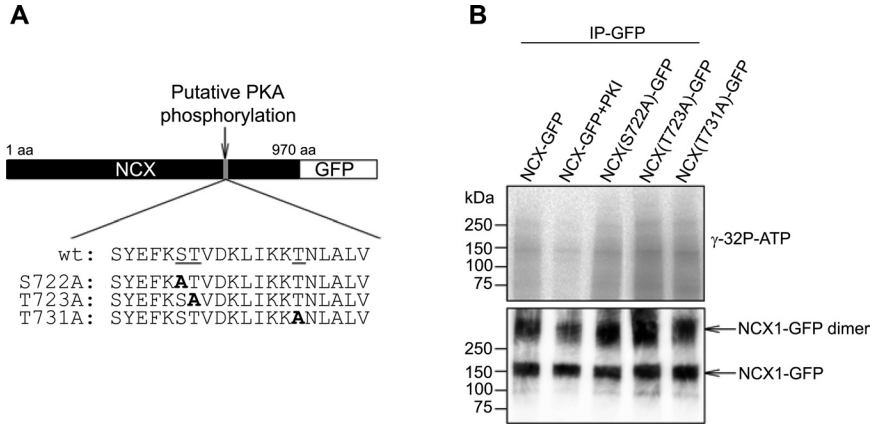

Fig. 3. Phosphorylation analysis of alanine substituted NCX1-green fluorescent protein (GFP). *A*: schematic illustration of the putative PKA phosphorylation sites (underlined) and alanine substitutions thereof (bold). *B*: immunocomplexes of NCX1-GFP, NCX1 (S722A)-GFP, NCX1 (T723A)-GFP, and NCX1 (T731A)-GFP were subjected to PKA phosphorylation with purified PKA-C and [γ-^{32}P]ATP, analyzed by SDS PAGE, and signal detected with BAS. Similar amounts of NCX-GFP-fusion proteins were immunoprecipitated as shown in *bottom*.

with the in vitro PKA phosphorylation site (Fig. 4*A*, boxed area). By immunoblotting, the antibody recognized protein bands of 120 and 140 kDa in nonboiled extract (*bottom* of Fig. 5, *A* and *B*, 37°C), whereas the 140-kDa was the major band in boiled extracts (96°C). The observed sizes are dependent on intramolecular disulfide bridges and are consistent with those previously reported for NCX1 (31). Second, a commercial phospho-threonine (pThr)-specific antibody, a PKA substrate antibody, and a custom-made pThr731-NCX1-specific antibody strongly recognizing synthetic pThr731-NCX peptides were identified (Fig. 4, *B*, *C*, and *D*, respectively, *top*, underlined sequences). Neither phospho-specific antibody cross-reacted with nonphosphorylated NCX1 (*bottom* of Fig. 4, *B*, *C*, and *D*).

To confirm this finding in a more physiological context, isolated neonatal rat cardiomyocytes were stimulated with forskolin or isoproterenol to activate PKA. NCX1 was immunoprecipitated with the custom-made NCX1 antibody. Phosphorylation was measured by immunoblotting using the two phospho-specific antibodies identified above. Although several PKA substrate-specific proteins were observed in the lysate after activation, no phosphoprotein was observed in the range of 120–140 kDa (Fig. 5*A*, *middle*). A p-Thr-specific protein of ~140 kDa coprecipitated with NCX1 (*top, right*) but was not detected with the anti-PKA substrate (*middle*, *right*) suggesting that the protein is phosphorylated by another kinase. Careful inspection revealed that the 140-kDa p-Thr-specific protein did not overlap with the NCX protein (*bottom panels*).

Furthermore, LV lysate isolated from rat was treated with forskolin, IBMX (PDE inhibitor that blunts cAMP degradation), and phosphatase inhibitors (cyclosporine A and NaF) to secure strong and consistent PKA activity. Phospho-proteins corresponding to molecular masses 140–160 kDa coimmunoprecipitated with NCX1 (Fig. 5*B*, PKA substrate, *middle*) but did not correspond to NCX1 (*bottom*). The phospho-proteins were also not recognized by the phospho-Thr-specific antibody (*top*). Noteably, a phospho-protein of ~70 kDa coimmunoprecipitated with NCX (Fig. 5*B*, *top* and *middle*) and partly overlapped with a 70-kDa proteolytic fragment of NCX (*bottom*). However, phosphoprotein levels were not increased after

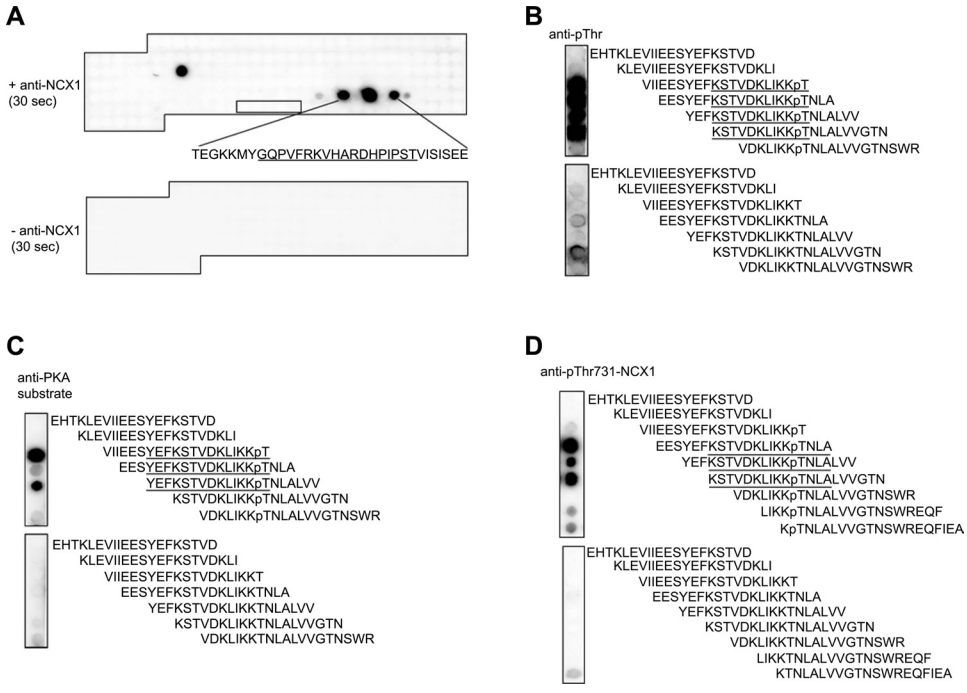

Fig. 4. Epitope mapping of antibodies. *A*: specificity of a custom-made NCX1 antibody. Residues important for antibody binding were identified by overlaying an array of immobilized NCX1 20-mer peptides with anti-NCX1 (*top*). The given amino acids are relevant for antibody binding and underlined amino acids indicate the core epitope (peptide used for immunization: 655-GQPVFRKVHARDHPIPST-672). Immunoblotting without incubation with primary antibody was used as a negative control (*bottom*). *B–D*: epitope mapping of phospho-specific antibodies against synthetic pThr731-NCX1 peptides. Residues important for binding were identified by overlaying an array of immobilized phosphorylated (*top*) or nonphosphorylated NCX1 20-mer peptides (*bottom*) with anti-phospho-threonine (anti-pThr) (*B*), anti-PKA substrate (*C*), or custom-made anti-pThr731-NCX1 (*D*). Underlined amino acids indicate the core epitopes (peptide used for immunization: 720-FKSTVDKLIKKpTNL-733).

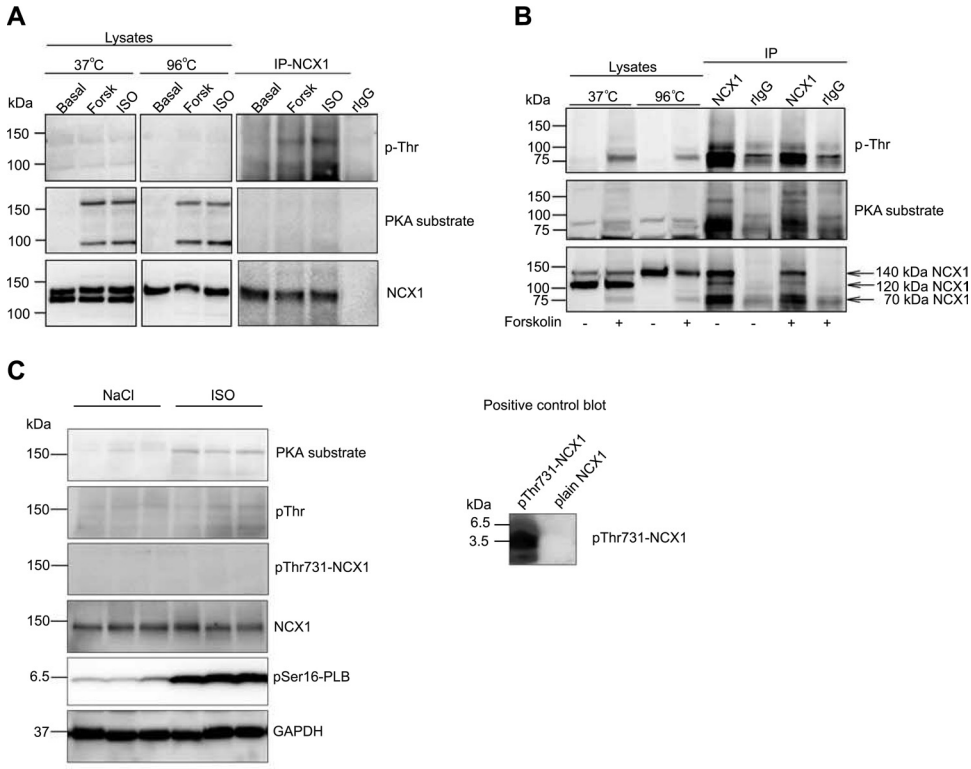

Fig. 5. Analysis of phospho-NCX1 level after PKA activation. *A*: basal, forskolin-, or isoproterenol (ISO)-stimulated neonatal rat cardiomyocytes were immunoprecipitated with the NCX1 antibody. Lysates and immunoprecipitates were analyzed by immunoblotting using anti-p-Thr (*top*), anti-PKA substrate (*middle*), or anti-NCX1 (*bottom*). *B*: LV lysate from rat treated with or without forskolin were immunoprecipitated with the NCX1 antibody. Lysates and immunoprecipitates were analyzed by immunoblotting using anti-p-Thr (*top*), anti-PKA substrate (*middle*), or anti-NCX1 (*bottom*). Identical amount of rabbit IgG (rIgG) was used as negative control (*A*, *B*). *C*: *left*; left ventricle homogenates from adult rats infused with Iso or NaCl (control). The homogenates were analyzed by immunoblotting using anti-PKA substrate (*top panel*), anti-pThr (*second panel*), pThr731-NCX1 (*third panel*), anti-NCX1 (*fourth panel*), anti-pSer16-PLB (*fifth panel*), or anti-GAPDH (*bottom panel*) as a loading control. *Right*: the pThr731-NCX1 peptide and the plain NCX1 peptide were analyzed by immunoblotting using anti-pThr731-NCX1.

forskolin stimulation, suggesting that the 70-kDa protein is phosphorylated by another kinase. Thus our data show no phosphorylation of endogenous NCX1 after PKA activation, suggesting the in vitro phosphorylation site in the full-length NCX1 protein to be inaccessible to the kinase.

Finally, we investigated whether NCX could be phosphorylated after in vivo β-adrenergic stimulation. Adult rats were infused with isoproterenol or sodium chloride (as control). Consistent with the above results, no NCX1 phosphorylation was observed with anti-PKA substrate, anti-pThr, or anti-pThr731-NCX1 (Fig. 5*C*, *top three panels*). The 150-kDa PKA substrate-specific protein observed after isoproterenol stimulation (*top panel*) did not overlap with the NCX protein (*fourth panel*). Phospho-serine 16 phospholamban (pSer16-PLB) was used as positive control for β-adrenergic activation (*fifth panel*) and GAPDH as loading control (*bottom panel*). A positive control blot showed that anti-pThr731-NCX1 strongly recognized the pThr731-NCX1 peptide but not the plain NCX1 (Fig. 5*C*, *right*). Collectively, these results provide strong evidence that full-length NCX1 is not a direct substrate of PKA.

No PKA phosphorylation of a 70-kDa proteolytic NCX1 fragment. Calpain or caspase-3 cleavage of endogenous NCX1 resulted in a fragment of ~70 kDa (Fig. 6, *A* and *B*, respectively) consistent with previously observations (1, 27). To examine whether the in vitro phosphorylation site could be exposed after cleavage, immunoprecipitated wild-type and alanine-substituted NCX1-GFP-fusion proteins (S722A, T723A, and T731A) expressed in HEK293 cells, were analyzed for their ability to be phosphorylated after cleaveage. No γ-^{32}P-labeled positive proteins of 70 kDa were observed for wild-type or mutated NCX1-GFP after calpain or caspase-3 cleavage (Fig. 6, *C* and *D*, respectively). As shown in Fig. 6, *C* and *D*, *bottom panels*, equal amounts of the 70-kDa proteolytic fragment were precipitated. These data suggest that the PKA phosphorylation site remains inaccessible in the 70-kDa proteolytic NCX1 fragment.

DISCUSSION

We have conducted a detailed examination of PKA phosphorylation of the cardiac NCX1 protein. By bioinformatic analysis and extensive use of NCX1-derived peptides, we identified a novel in vitro PKA phosphorylation site at threonine 731 in the cytoplasmic region of rat NCX1. The site (IKKT) was conserved in mammalian species including the human, rat, mouse, rabbit, dog, and cow. Interestingly, the site was substituted with an alanine in species such as squid, *Drosophila*, and *C. elegans*. Importantly, we observed that this phosphorylation site was not accessible in full-length NCX1 or following cleavage by calpain or caspase. These data suggest that NCX1 is not a direct target for PKA phosphorylation.

The regulation of NCX1 phosphorylation is not well defined, and several reports have suggested that NCX1 from different species, including the rat, pig, and dog, can be phosphorylated by PKA in vitro (16, 36, 44). Our findings from peptide arrays are consistent with these reports, and the notion that the cytoplasmic domain in cardiac NCX1 is a good substrate for PKA (34). Immobilized peptide arrays have been proven to be powerful tools for identification of kinase substrates. Unspecific binding of negatively charged [γ-^{32}P]ATP to positively charged peptides is often observed in such high-density arrays (37), but false positives can easily be ruled out by introducing control peptides lacking the phospho-acceptor residue. By using immobilized arrays with overlapping 20-mer synthetic NCX1 peptides spanning the large intracellular loop, we found that threonine 731 (IKKT) in the NCX1 protein is highly

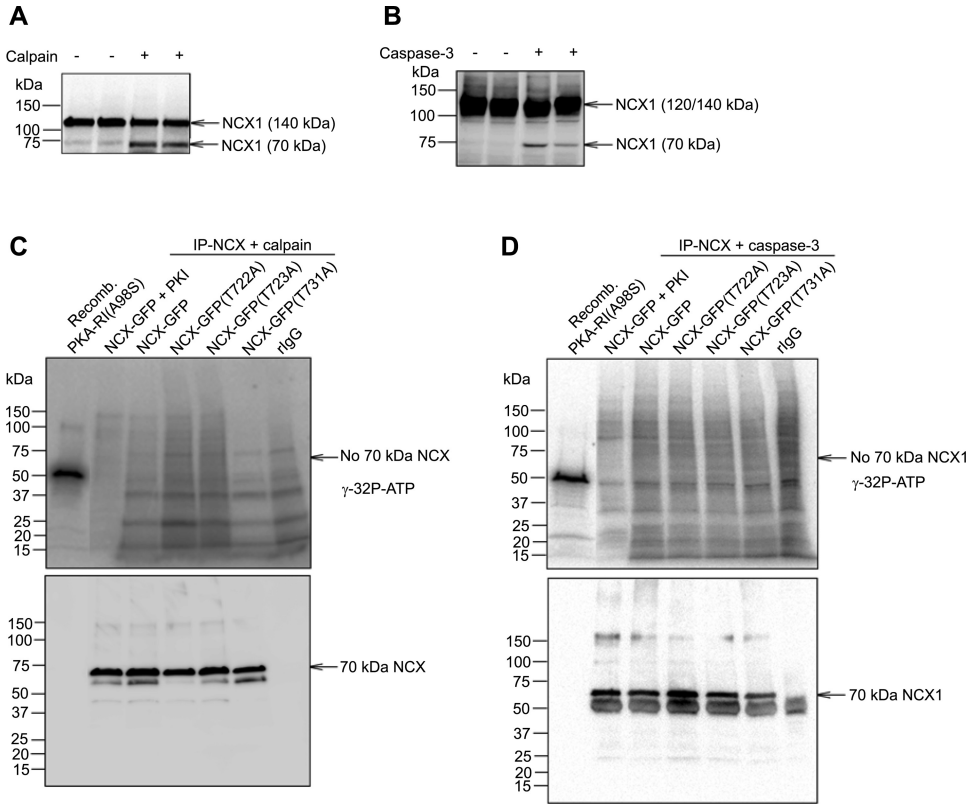

Fig. 6. Analysis of PKA phosphorylation of NCX1 after calpain or caspase-3 cleavage. LV tissue lysates were treated with or without calpain (*A*) or caspase-3 (*B*). The lysates were analyzed by immunoblotting using anti-NCX1. Immunocomplexes of NCX1-GFP, NCX1 (S722A)-GFP, NCX1 (T723A)-GFP, and NCX1 (T731A)-GFP were subjected to PKA phosphorylation with [γ-^{32}P]ATP after calpain (*C*) or caspase-3 cleavage (*D*), analyzed by SDS PAGE, and signal detected with BAS. Similar amounts of NCX-GFP-fusion proteins were immunoprecipitated as shown in *C* and *D* (*bottom*). PKI was used as a control for specific PKA phosphorylation. PKA-RI (A98S) (see Ref. 3) was used as positive control for phosphorylation. Identical amount of rabbit IgG (rIgG) was used as negative control (*C*, *D*).

phosphorylated by PKA in vitro and that substitution of threonine by alanine (T731A) abolished the phosphorylation. Threonine 731 (IKKT) was phosphorylated to a similar extent as the serine in the consensus PKA site RRAS. Thus threonine 731 is a robust PKA phosphorylation site in NCX1 protein in vitro. However, we did not observe phosphorylation at Ser389 in the consensus site KAVS, although previous reports have suggested this to be a site of PKA phosphorylation in canine and frog NCX1 (17, 25).

We further investigated whether full-length NCX1 protein could be phosphorylated by PKA by analyzing immunoprecipitated NCX-GFP. We additionally employed epitope-mapped phospho-specific antibodies to examine the effects of forskolin or isoproterenol treatment on isolated neonatal rat cardiomyocytes, LV lysates, and adult rats. None of these approaches detected NCX1 phosphorylation mediated by PKA. However, a 70-kDa proteolytic fragment of NCX1 was observed and appeared to be strongly phosphorylated. We therefore examined whether protein degradation of NCX1 could contribute to NCX1 phosphorylation. NCX1 has been identified as a substrate for Ca^{2+}-activated proteases such as calpains and caspases (1, 19, 35). Calpain or caspase-3 cleavage of endogenous rat NCX1 resulted in a fragment of ~70 kDa, consistent with previous observations (1, 27). To examine whether the in vitro phosphorylation site could be exposed to PKA after cleavage, immunoprecipitated NCX1-GFP fusion proteins were digested with calpain and caspase-3 before PKA phosphorylation analysis. However, we could not observe any phosphorylation of the 70-kDa proteolytic NCX1 fragment in either wild-type or mutated NCX1-GFP.

Results from functional studies of NCX in isolated cardiomyocytes have been conflicting (46). While some have observed increased NCX current after treatment with PKA activators (26, 33, 44), others have not observed any change (9, 20, 21). We were unable to detect PKA-dependent phosphorylation of the full-length NCX1 protein and suggest that the discrepancy between previous functional studies likely results from experimental differences in ionic homeostasis. NCX function is importantly controlled by local levels of Na^+ and Ca^{2+}, and these can be markedly altered during PKA phosphorylation and activation of the L-type Ca^{2+} channel (18, 23), ryanodine receptor (22, 42), the Na^+-K^+-ATPase (40), and the SERCA (5). Importantly, phospholemman another PKA substrate, has recently been shown to interact with and to have an inhibitory effect on NCX1 activity when phosphorylated (45). Thus indirect effects on NCX currents are expected following PKA activation, and these may vary depending on experimentally set intra- and extracellular ionic concentrations.

Whereas our study does not support a role for PKA phosphorylation of the NCX protein, it remains to be determined whether other kinases play a functional role. Indeed, we observed that a proteolytic NCX fragment of 70 kDa was phosphorylated in LV lysates, but that phosphorylation was not increased during forskolin stimulation, suggesting involvement of another kinase. Alternative kinases may contribute to the indirect effects of isoproterenol reported in isolated cardiomyocyte experiments. NCX1 is suggested to be phosphorylated by protein kinase C (PKC) (15, 16) and regulated after treatment with PKC activators (15, 16, 41, 47). Importantly, PKC is part of the NCX1 macromolecular complex along with different phosphatases (36). PKA-dependent alterations in Ca^{2+} homeostasis might also promote CaMKII activation and NCX phosphorylation; however, such a role of CaMKII remains to be elucidated. Future work will be aimed at examining non-PKA-

dependent phosphorylation of NCX using the techniques employed in the present study. In conclusion, our results indicate the presence of a novel in vitro PKA phosphorylation site on NCX1, which is not accessible to the kinase in either full-length protein or in a 70-kDa proteolytic fragment. These findings suggest that NCX1 might not be a direct physiological substrate of PKA. However, we cannot exclude that PKA phosphorylates NCX1 under other circumstances nor can our data exclude that NCX1 activity is indirectly modulated by PKA.

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DISCLOSURES

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

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