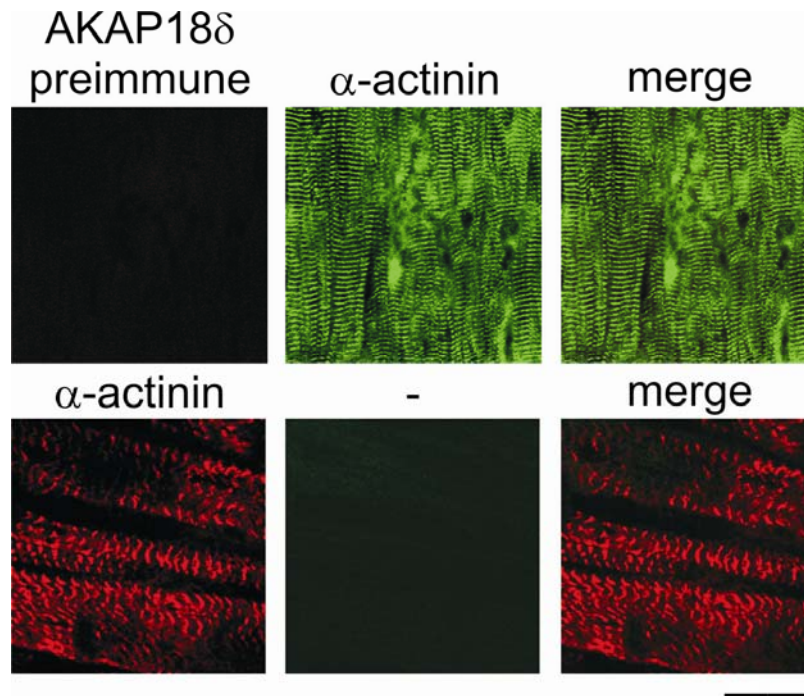
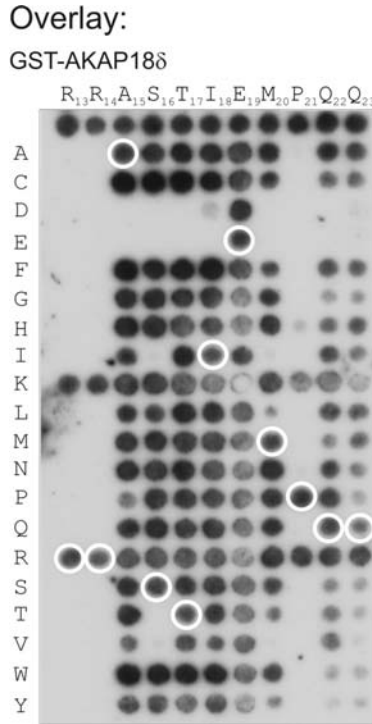


**SUPPLEMENTARY INFORMATION**  
to  
**Lygren et al. “AKAP-complex regulates  $Ca^{2+}$  reuptake into heart sarcoplasmic reticulum”**

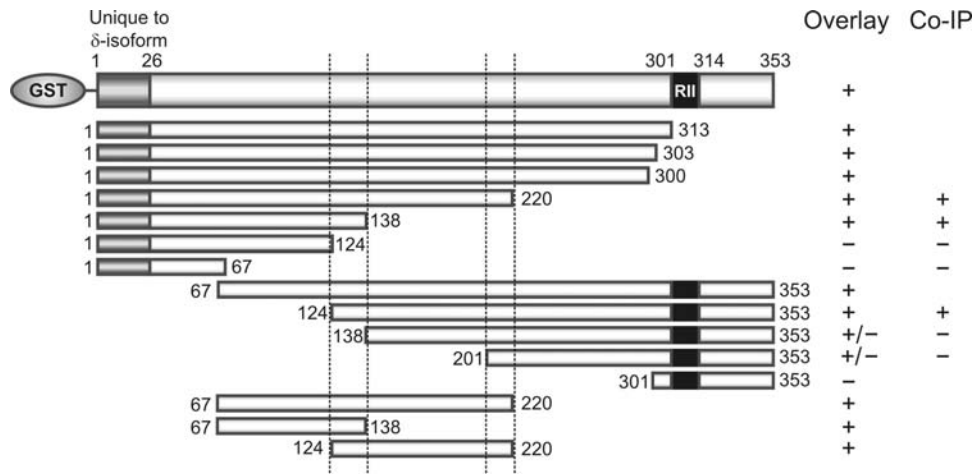
**SUPPLEMENTARY FIGURES**



**Supplementary Fig S1. AKAP18δ immunostaining specificity controls.** In order to test for specificity and rule out cross-bleeding between Cy3 (red) and Cy5 (green) channels in immunohistochemical analyses, parallel controls with preimmune serum and without primary antibodies were conducted with all immunostainings. For example, rat heart tissue was incubated with AKAP18δ preimmune serum (rabbit) and monoclonal anti-α-actinin antibodies (upper row). Cy3-conjugated anti-rabbit IgG or Cy5-conjugated anti-mouse IgG (1:600) were used as secondary antibodies. Note: No AKAP18δ signal appeared and Cy5 signals were not detectable in the Cy3 channel. In order to control for bleeding from the Cy3- into the Cy5 channel primary anti- AKAP18δ antibody was omitted (lower row). As secondary antibody Cy5-conjugated anti-rabbit IgG was used. No Cy3 signals from parallel staining with α-actinin and Cy3-conjugated anti-mouse IgG were detectable in the Cy5 channel. Scale bar, 20 μm.



**Supplementary Fig S2. Analysis of AKAP18δ interacting site in PLB.** Two-dimensional arrays of 220 peptide derivatives of PLB (13-23) where each residue in the native peptide (given by single-letter code above the array) was systemically substituted with all natural amino acids (single-letter code at the left of the array) were spot-synthesized and analyzed for interaction with AKAP18δ by GST-AKAP18δ overlay. The first row corresponds to the native sequence.



**Supplementary Fig S3. Deletional mapping of the PLB binding domain in AKAP18δ.** Overlapping peptides representing PLB were spot-synthesized on cellulose membranes

and the arrays overlaid with truncated GST-AKAP18 $\delta$  proteins as indicated. In addition, GFP-PLB and AKAP18 $\delta$  truncated proteins were co-expressed in HEK293 cells to analyze Flag-AKAP18 $\delta$ -PLB binding *in situ* and interaction assessed by co-immunoprecipitation (IP) (left and right columns, respectively; +/- indicate binding). GST alone was used as a negative control (not shown). **Note:** *In vitro* binding of various constructs outlines amino acids 124 to 138 and amino acids 201 to 220 of AKAP18 $\delta$  as important for binding (dotted lines). GFP immunoprecipitation demonstrated that AKAP18 $\delta$  constructs covering amino acids 1-220 containing both binding regions and 1-138 containing the N-terminal binding region were co-immunoprecipitated with GFP-PLB, but the interaction of the AKAP18 $\delta$  138-353 fragment with PLB appeared weaker than that of AKAP18 $\delta$  1-220, suggesting that both domains might co-operate in binding *in vivo*. The mapped binding regions are not unique for the  $\delta$  isoform of AKAP18, but are also present in the AKAP18 $\gamma$  isoform (Henn *et al.*, 2004). Thus, we can not exclude that AKAP18 $\gamma$  also binds PLB. However, immunofluorescence microscopy analyses with an antibody directed against both the AKAP18 $\gamma$  and AKAP18 $\delta$  isoforms decorated m-lines and z-lines whereas the AKAP18 $\delta$ -specific antibody decorated z-lines only, indicated that AKAP18 $\gamma$  has a distribution that is only partially overlapping with SERCA2 or PLB (data not shown).

## METHODS

### Heart subcellular fractionation

Rat hearts were fractionated according to a protocol described elsewhere (Kapiloff *et al.*, 2001) with some modifications. Specifically, two rat hearts (Pel-Freeze Biologicals, Rogers, AR, USA) were disrupted using a mortar in 20 ml buffer B (10 mM Hepes pH 7.4, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 1 mM benzamidine, 5 mM EDTA, protease inhibitors (Complete Mini, EDTA-free tablets, Roche)) with 0.32 M sucrose. Whole heart homogenate was filtered through cheesecloth, before low-speed centrifugation at 3,800 g for 20 min. The supernatant fraction (S1) was re-centrifuged at 100,000 g for 1 h. The resulting pellet (P2), containing SR, Golgi apparatus and plasma membrane, was resuspended in 2 ml buffer B with 0.32 M sucrose. SR was obtained by sucrose gradient centrifugation of P2 (layered on top of 8 parts 24%, 6 parts 40%, 2 parts 50% sucrose in 5 mM Hepes buffer) at 100,000 g for 90 min. SR then forms a layer at the interface between 24% and 40% sucrose and was typically recovered in fractions 9-13 from the top. All centrifugation steps were carried out at 4 °C.

### Heart homogenate

The lower side of the left ventricle of fresh rat heart was lysed in 2-5 ml lysis buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Tween 20) containing 8  $\mu$ l/ml protease inhibitor stock solution (2 mg/ml soybean trypsin inhibitor, 1.43 mg/ml trasylol (aprotinin), 100 mM benzamidine, 500  $\mu$ M phenylmethanesulfonyl fluoride (PMSF)). Samples (1 ml each) were homogenized in a glass/teflon homogenizer (10 strokes, 1250 rpm) and centrifuged at 24,000 g for 20 min at 4°C. Supernatants were collected for further analyses.

### **Cardiac myocyte preparation**

Ventricles were isolated from 1-3 day or 8-12 week old Wistar rat hearts by four cycles of enzymatic digestion at 37 °C for 10 min using 0.48 mg/ml collagenase type II (Biochrom AG, Germany) and 0.6 mg/ml pancreatin (Sigma, Germany), and subsequent centrifugation (170 x g, 5 min). The cells of the final pellet were suspended in DMEM:M199 (4:1) medium supplemented with 10% horse serum (Invitrogen, Gibco) and 5% fetal calf serum (Invitrogen, Gibco) and seeded for 1 h on tissue culture plates to deplete fibroblasts. Non-adherent neonatal myocytes were plated on 1% (w/v) gelatin pre-coated plates or glass cover slips pre-coated with 0.5 mg/ml laminin (Rochè, Mannheim, Germany). After 24 h, the medium was changed to low serum medium (DMEM:M199) containing 4% horse serum. Cells were lysed in PBS with 1% Triton X-100 and protease inhibitors (Complete Mini, EDTA-free tablets, Roche) or processed for immunofluorescence microscopy. Isolated adult myocytes were plated on laminin-coated glass cover slips (4µg/cm<sup>2</sup>, Roche) in plating medium (64 % DMEM, 18 % M-199, 10% Horse serum, 5% Fetal calf serum, 2 mM glutamine). After 16 h, the medium was changed to serum-free medium cells subjected to incubation with peptides and immunostaining..

### **RII-overlay**

RII-overlays were conducted as described (Hausken *et al.*, 1998), using <sup>32</sup>P-labeled recombinant murine RIIα. Briefly, the membrane was blocked in blotto (5% [w/v] non-fat dry milk plus 0.1% bovine serum albumin (BSA) in Tris-buffered saline (TBS)). Purified recombinant RII (4 µg) was radiolabeled with purified catalytic subunit (C) of PKA (0.02 µg/µl) and [ $\gamma$ -<sup>32</sup>P] ATP (1.4 µCi µl<sup>-1</sup>) in 50 mM MOPS, 50 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM DTT, pH 6.8 and separated from free <sup>32</sup>P-ATP by gel filtration (G-50 sepharose) and incubated in blotto with cAMP and cold ATP to avoid binding to the C subunit and unspecific phosphorylation. The membrane was washed in TBS with 0.1% Tween-20 (TBST) and signals detected by autoradiography.

### **Immunoblot analysis**

Cell lysates and immunocomplexes were analyzed on a 10%, 12.5% or 4-20% SDS/PAGE and blotted onto PVDF membranes. The filters were blocked in 5% non-fat dry milk in TBST for 30 min at RT, incubated 1 hour at RT or 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 Supersignal West Pico substrate (Pierce, Rockford, IL, USA) or Lumi-Light Western Blot substrate solution (Roche Diagnostics).

### **Immunohistochemistry**

Rats were sacrificed; the left ventricles of the hearts were removed and cut into smaller fragments using a razor blade. The tissue was shock frozen in liquid nitrogen, before embedded in Tissue Embedding Medium (Jung, Tissue freezing medium, Leica Instruments GmbH, Nussloch, Germany) mounted in the cryostat and cut into sections (4 µm; Cryostat CM 3000 from Lyeka) using a D-knife while the tissue temperature was -20°C. Sections (3 to 4) were transferred from the knife to each glass slide (Menzel, Superfrost Plus, Germany), and fixed with 2.5% paraformaldehyde in sodium cacodylate

buffer (100 mM sodium cacodylate and 100 mM sucrose, pH 7.4) for 30 min. After three washes with phosphate-buffered saline (PBS), cells were permeabilized in PBS containing 0.1% Triton X-100 for 5 min and rewashed three times. Blocking was carried out in blocking solution (0.3 ml 45% fish skin gelatine/100 ml PBS) in a humidifying chamber by incubation for 45 min at 37°C. After three washes with PBS (10 min each wash), antibody diluted in blocking solution was added. The slides were incubated in a humidifying chamber for 45 min at 37°C, before washing three times (10 min each wash) with cold PBS, and subsequently incubated in a humidifying chamber for 45 min at 37°C with secondary antibody. Coverslips were mounted in Immu-Mount (Thermo Shandon, Thermo Electron Corporation, Dreieich, Germany) according to the manufacturer instructions. Signals were visualized by confocal microscopy on a Zeiss laser scanning microscope LSM 510meta (Zeiss, Jena, Germany).

### **Immunogold electron microscopy**

Immunogold electron microscopy was carried out as described (Henn *et al.*, 2004). Hearts were obtained from neonatal rats, fixed (0.25% glutaraldehyde, 3% formaldehyde), cryosubstituted in a Leica AFS freeze-substitution unit and embedded in LR-White. The samples were sequentially equilibrated over 4 days in methanol at temperatures gradually increasing from -90°C to -45°C. The samples were infiltrated with LR-White for 72 h at -20°C, and polymerized for 1 h at -20°C and 2 h at 4°C. Sections (60 nm) were cut on a Reichert Ultracut S, placed on nickel grids, blocked and immunogold labeled as described elsewhere (Berryman and Rodewald, 1995). The sections were incubated with primary antibodies, washed with TBS with 1% normal serum and incubated with secondary antibodies coupled to 10 to 18 nm gold grains (British Biocell or Dianova). The sections were stained with uranyl acetate and lead citrate and analyzed with a 80 kV electron microscope (902A, LEO, Obercochem, Germany) equipped with a slow scan CCD camera (Megaview III, Soft Imaging System, Germany) and the analySIS software (Soft Imaging System).

### **Immunoprecipitation from pooled rat heart SR fractions and total homogenates**

SR fractions or total homogenates (500 µg) were incubated with antibodies and protein A/G agarose beads (Invitrogen Corporation, CA) overnight at 4°C. Immunocomplexes were washed four times in lysis buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 or 0.5% Tween 20, protease inhibitors) boiled in SDS loading buffer, resolved by SDS/PAGE and detected by immunoblotting.

### **Antibodies**

Immunoblotting and immunoprecipitation were carried out with antibodies against AKAP18δ (Henn *et al.*, 2004), GFP (BD Biosciences, CA, US), PKA RIα, RIIα and RIIβ (BD Transduction Laboratories, Lexington, KY, USA), Cα, SERCA2 and IP3R-II (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), NCX (Thomas *et al.*, 2003), Calsequestrin and PLB (Upstate Biotechnology Inc., Lake Placid, NY, USA), RYR (Affinity BioReagents, CO, USA), pSer<sup>16</sup>-PLB (Badrilla Ltd., West Yorkshire, UK), GST (Amersham Pharmacia biotech). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgGs was used as a secondary antibody (Jackson ImmunoResearch). Rabbit or mouse gamma globulin (Jackson ImmunoResearch Laboratories Inc., PA, USA) or

preimmune serum was used as negative control. For immunostaining, antibodies against  $\alpha$ -actinin (Sigma-Aldrich, Saint Louis, Missouri, USA), SERCA2 (Alexis Biochemicals), RII $\alpha$ , PLB, AKAP18 $\delta$  plus Cy3- or Cy5-conjugated anti-rabbit and anti-mouse IgG were used. For immunogold electron microscopy, antibodies against AKAP18 $\delta$ , PLB and SERCA2 (ABR, Affinity Bioreagents, Dianova, Germany) were used.

### **Transfection of HEK293 cells**

HEK293 cells at 60-80% confluency were transfected with 10  $\mu$ g of plasmid DNA per 567 mm<sup>2</sup> culture dishes using Lipofectamin2000 (Invitrogen) according to the manufactures instructions. Cells were lysed 24 h after transfection in lysis buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Tween 20) with protease inhibitors (Complete Mini EDTA free tablets, Roche).

### **Protein expression and purification**

GST-AKAP18 $\delta$  and GST were expressed in *E.coli* BL21 by IPTG induction. The GST-AKAP18 $\delta$  or GST containing pellet was incubated in lysis buffer (10 mM MOPS pH 6.5, 100 mM NaCl, protease inhibitors (Complete Mini, EDTA-free tablets, Roche)) and sonicated (UP400s Ultraschall processor) for 1 min in three intervals at 0°C. After centrifugation, the supernatant was incubated with glutathione-agarose beads (Sigma) and rotated overnight at 4°C. The recombinant protein bound to the beads was washed two times in lysis buffer thereafter two times in washing buffer (5 mM MOPS pH 6.5, 0.5 M NaCl) and finally two times in lysis buffer. The recombinant protein was eluted with 20 mM L-Glutathione (reduced, in 50 mM Tris-HCl pH 8.4 150 mM NaCl) at 4°C overnight before dialysis into PBS.

### **cAMP pull down**

Pooled SR fractions were subjected to cAMP pull down experiment using Rp-8-AHA-cAMP agarose beads (antagonist that does not dissociate the C subunit from the PKA holoenzyme, Biolog Life Science Institute, Germany) in homogenisation buffer (20 mM Hepes pH 7.4, 20 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 1 mM DTT, protease inhibitors). Proteins bound to cAMP-agarose were washed four times in washing buffer (10 mM Hepes pH 7.4, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.1% NP-40, 1 mM DTT, protease inhibitors) and eluted with 75  $\mu$ M cAMP.

### **Spot synthesis of peptide arrays and overlay experiments**

PLB peptide arrays were spot-synthesized on cellulose membranes by using Multi pep automated peptide synthesizer (Intavis Bioanalytical Instruments AG, Germany) as described (Frank, 1992). Interaction of spotted peptides with GST-AKAP18 $\delta$  or GST was tested by overlaying the membranes with 1  $\mu$ g/ml of recombinant protein in TBST. Bound recombinant protein was detected with anti-GST.

### **Peptide synthesis**

Peptides were synthesized and purified to >80% purity (in house Multi pep automated peptide synthesizer and HPLC) using standard f-moc chemistry.

PLB-Arg<sub>9-11</sub>: RRASTIEMPQQ-R<sub>9-11</sub>

Arg<sub>9</sub>-PLB: R<sub>9</sub>-RRASTIEMPQQ

Arg<sub>9</sub>-pSer<sup>16</sup>-PLB: R<sub>9</sub>-RRApSTIEMPQQ

### siRNA

siRNA oligos used were synthesized in house (Applied Biosystems DNA/RNA synthesizer mod. 394) or by Dharmacon RNA Technologies (Chicago, IL) The sequences of the siRNAs shown are:

AKAP18δ: 5' GGG AGA AAU AGA UGC CAA UAA 3'  
5' AUU GGC AUC UAU UUC UCC CGC 3'

Control: 5' GGG ACA AAU ACA UGG CAA UAA 3'  
5' A UUG CCA UGU AUU UGU CCC GC 3'

The AKAP18δ siRNA was tested in HaCaT cells transfected with AKAP18δ before examining effects in cardiomyocytes and inhibited expression by >80%. Similar observations were made with two other siRNAs directed against AKAP18δ, indicating that the observations made are not off-target effects. In order to identify cardiac myocytes that received both the siRNA and the FRET-based Ca<sup>2+</sup> sensor Cameleon D1ER by transfection, siRNA was labeled with Cy3 and effects on Ca<sup>2+</sup> assessed in double positive cells.

### Calcium imaging

Rat neonatal cardiac myocytes for the peptide experiment were transfected (Transfectin reagent, Bio-Rad) with the FRET-based Ca<sup>2+</sup> sensor Cameleon D1ER targeted to the SR (Palmer *et al.*, 2004). After 36-48 h the response to a 10 mM caffeine pulse (1s) generated by a pneumatic picopump PV820 (WPI) was recorded in control cells or cells pre-treated with 25 μM peptide (PLB-Arg<sub>11</sub>) for 40 min and/or 10 μM NE and 100 μM IBMX for 20 min. The experiments were performed in perfusion with a modified Ringer's solution containing 1 mM Ca<sup>2+</sup> and 1 mM glucose.

For the siRNA experiments, rat neonatal cardiac myocytes were transfected as indicated above with a FRET-based Ca<sup>2+</sup> sensor cameleon targeted to the SR (D1ER) in the absence or in the presence of 50 nM siRNA or control siRNA. Real-time imaging experiments were performed 48 h post-transfection in a modified Ringer's solution containing 1 mM glucose, 30 mM KCl and either 300 μM EGTA or 3 mM Ca<sup>2+</sup>. Cells were imaged (1 frame/10s) in the presence of 50 μM of the SERCA2 inhibitor 2,5-Di-*tert*-butylhydroquinone (BHQ; Sigma-Aldrich) in the presence of 300 μM EGTA to monitor the release of Ca<sup>2+</sup> from the SR. BHQ was subsequently washed away with a solution containing 1 mM glucose, 30 mM KCl and 300 μM EGTA. In order to monitor Ca<sup>2+</sup> reuptake into the SR, cells were subsequently imaged at 1 frame/250 ms in the presence of 3 mM Ca<sup>2+</sup>. In cells treated with 10 μM NE, the beta-agonist was added at the start of the washing out phase and for the entire duration of the experiment. The value of the time constant  $\tau$  was calculated by fitting the recovery phase in the curve of Ca<sup>2+</sup> reuptake by using the exponential function  $f(t) = \sum_{i=1}^n A_i e^{-t/\tau_i} + C$ .

## REFERENCES

Berryman M and Rodewald R (1995) Beta 2-microglobulin co-distributes with the heavy chain of the intestinal IgG-Fc receptor throughout the transepithelial transport pathway of the neonatal rat. *J Cell Sci*, **108** ( Pt 6), 2347-2360.

Frank R (1992) Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron*, **48**, 9217-9232.

Hausken ZE, Coghlan VM, and Scott JD (1998) Overlay, ligand blotting, and band-shift techniques to study kinase anchoring. *Methods Mol Biol*, **88**, 47-64.

Henn V, Edemir B, Stefan E, Wiesner B, Lorenz D, Theilig F, Schmitt R, Vossebein L, Tamma G, Beyermann M, Krause E, Herberg FW, Valenti G, Bachmann S, Rosenthal W, and Klusmann E (2004) Identification of a novel A-kinase anchoring protein 18 isoform and evidence for its role in the vasopressin-induced aquaporin-2 shuttle in renal principal cells. *J Biol Chem*, **279**, 26654-26665.

Kapiloff MS, Jackson N, and Airhart N (2001) mA-KAP and the ryanodine receptor are part of a multi-component signaling complex on the cardiomyocyte nuclear envelope. *J Cell Sci*, **114**, 3167-3176.

Palmer AE, Jin C, Reed JC, and Tsien RY (2004) Bcl-2-mediated alterations in endoplasmic reticulum Ca<sup>2+</sup> analyzed with an improved genetically encoded fluorescent sensor. *Proc Natl Acad Sci U S A*, **101**, 17404-17409.

Thomas MJ, Sjaastad I, Andersen K, Helm PJ, Wasserstrom JA, Sejersted OM, and Ottersen OP (2003) Localization and function of the Na<sup>+</sup>/Ca<sup>2+</sup>-exchanger in normal and detubulated rat cardiomyocytes. *J Mol Cell Cardiol*, **35**, 1325-1337.