Identification of PDZ Domain Containing Proteins Interacting with Ca\textsubscript{v}1.2 and PMCA4b

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Received 30 November 2012; Accepted 25 December 2012

Academic Editors: T. Yazawa, N. Zambrano, and Y. Zhang

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PDZ (PSD-95/Disc large/Zonula occludens-1) protein interaction domains bind to cytoplasmic protein C-termini of transmembrane proteins. In order to identify new interaction partners of the voltage-gated L-type Ca\textsuperscript{2+} channel Ca\textsubscript{v}1.2 and the plasma membrane Ca\textsuperscript{2+} ATPase 4b (PMCA4b), we used PDZ domain arrays probing for 124 PDZ domains. We confirmed this by GST pull-downs and immunoprecipitations. In PDZ arrays, strongest interactions with Ca\textsubscript{v}1.2 and PMCA4b were found for the PDZ domains of SAP-102, MAST-205, MAGI-1, MAGI-2, MAGI-3, and ZO-1. We observed binding of the Ca\textsubscript{v}1.2 C-terminus to PDZ domains of NHERF1/2, Mint-2, and CASK. PMCA4b was observed to interact with Mint-2 and its known interactions with Chapsyn-110 and CASK were confirmed. Furthermore, we validated interaction of Ca\textsubscript{v}1.2 and PMCA4b with NHERF1/2, CASK, MAST-205 and MAGI-3 via immunoprecipitation. We also verified the interaction of Ca\textsubscript{v}1.2 and nNOS and hypothesized that nNOS overexpression might reduce Ca\textsuperscript{2+} influx through Ca\textsubscript{v}1.2. To address this, we measured Ca\textsuperscript{2+} currents in HEK 293 cells co-expressing Ca\textsubscript{v}1.2 and nNOS and observed reduced voltage-dependent Ca\textsubscript{v}1.2 activation. Taken together, we conclude that Ca\textsubscript{v}1.2 and PMCA4b bind promiscuously to various PDZ domains, and that our data provides the basis for further investigation of the physiological consequences of these interactions.

1. Introduction

PDZ domains are protein interaction motifs that play a crucial role in cellular signaling and bind specifically to cytoplasmatic carboxyl (C-) terminal sequences of their interacting proteins, which often belong to transmembrane receptor and ion channel families. This motif typically spans 90–100 amino acids and was first found in three polypeptides: the mammalian protein postsynaptic density-95 (PSD-95), the Drosophila melanogaster epithelial tumor suppressor protein Discs Large (Dlg), and the mammalian epithelial tight junction protein Zonula occludens-1 (ZO-1) [1–7]. Typical ligands for PDZ domains are the high voltage-activated L-type calcium channel (LTCC) Ca\textsubscript{v}1.2 and the plasma membrane calcium ATPase (PMCA) 4b, both essential for calcium homeostasis in excitable and nonexcitable cells.

Voltage-gated calcium channels (Ca\textsubscript{v}) allow the cellular entry of calcium (Ca\textsuperscript{2+}) ions and initiate muscle excitation-contraction coupling, neurotransmitter release, gene expression, or hormone secretion. Ca\textsubscript{v}1.2 channels play a major role for the voltage-dependent Ca\textsuperscript{2+} influx. Its overexpression augmented Ca\textsuperscript{2+} influx into cardiomyocytes, thereby increasing cardiac contractile force [8, 9]. Further studies demonstrated the importance of Ca\textsubscript{v}1.2 in the heart. Homozygous Ca\textsubscript{v}1.2 knockout mice die before day 14.5 p.c., presumably because L-type channels are indispensable during heart development [10, 11]. Furthermore, mutations in the CACNA1C gene, which codes for the Ca\textsubscript{v}1.2 subunit, are causative for the Timothy syndrome. This disease is characterized by a multiorgan disorder with serious cardiac defects, sudden death, and other comorbidities [12, 13].

PMCA4b extrude Ca\textsuperscript{2+} from the cytoplasm into the extracellular space, and they, as primary ion pumps, are
regarded as essential for long term maintenance of low intracellular Ca\textsuperscript{2+}, which is a prerequisite for subsequent Ca\textsuperscript{2+}-dependent intracellular signaling and spatial changes in Ca\textsuperscript{2+} concentrations [14–16]. Mammalian PMCA are products of four genes (ATP2B1–ATP2B4), which share 80–90% sequence homology at the amino acid level in human, rat, and mouse [17]. In many cell types, PMCA are concentrated in caveolae [18–20] that are rich in receptors, signal transducers, effectors, and structural proteins, all of which are important for many signaling pathways and the maintenance of cytoskeletal networks [21, 22]. Differential splicing of PMCA RNA transcripts results in a variety of subtypes of these isoforms. More than 20 splice variants have been identified [17]. The C-termini of the b-splice variants of all PMCA isoforms is supposed to bind preferentially to type 1 PDZ domains. The human ETSV\textsuperscript{+} motif of the PMCA4b interacts with members of the membrane-associated guanylate kinase (MAGUK) family [17, 18], such as PSD-95/SAP90, SAP97/hDlg. SAP-102, PSD-93/Chapsyn-110 [18], and calcium/calmodulin-dependent serine protein kinase (CASK) [23], which contain one to six PDZ domains [24, 25]. MAGUKs are essential for organizing signaling complexes, and membrane protein trafficking [26]. PMCA4b interacts with PMCA-interacting single PDZ domain protein (PISP) [27], Na\textsuperscript{+}/H\textsuperscript{+} exchanger regulatory factor 2 (NHERF2) [28], and neuronal nitric oxide synthase (nNOS), thereby regulating enzyme activity [29]. Besides C-terminal mediated interactions, PMCA4b also interacts with other proteins via other domains, for example, via the second intracellular loop with the tumor suppressor Ras-associated factor 1, calcineurin, and α1-syntrophin [30–32]. So far, there are only few studies about the proteins that may possibly interact with Ca\textsubscript{1.2}. To gain more insight into the participating pathways, and the physiological roles of Ca\textsubscript{1.2} and PMCA4b, we searched for new interaction partners. In this study, we show that the Ca\textsubscript{1.2} motif (VSNL\textsuperscript{+}) interacts with the MAGUK protein CASK, and the membrane-associated guanylate kinase-inverted proteins 1, 2, 3 (MAGI-1, MAGI-2, MAGI-3), a subfamily of MAGUK proteins. MAGI-1, MAGI-2, MAGI-3 are also new interaction partners of PMCA4b. Interestingly, we discovered additional proteins that interact with Ca\textsubscript{1.2} as well as with PMCA4b. These are the microtubule-associated tectis specific serine/threonine kinase (MAST-205), the tight junction protein Zonula occludens 1 (ZO-1), Rho guanine exchange factor 11 and 12 (GEF11, GEF12), and Mnt2-2. In addition, we confirmed the lack of PMCA4b interactions with NHERF1 and NHERF2, and in contrast, the binding of Ca\textsubscript{1.2} to the PDZ domain of NHERF2/2. We further showed that the nNOS interacts with Ca\textsubscript{1.2}, thereby influencing transmembrane current activities.

Our results provide the basis for more detailed analyses of PMCA4b and Ca\textsubscript{1.2} channel regulation, specifically in dependence of the associated PDZ domain containing proteins.

2. Materials and Methods

2.1. Plasmid Constructs. Plasmids encoding Histidine (His)-tagged carboxyl termini of Ca\textsubscript{1.2α} (LTCC) and PMCA4b were made by standard molecular biology techniques. Codons for the final 10 (Ca\textsubscript{1.2α}) or 15 (PMCA4b) amino acids were cloned as PstI-XbaI fragments into the pEXP vector (containing a 6xHis tag, Panomics, Fremont, CA, USA) to produce His-tagged fusion proteins (pEXP-Ca\textsubscript{1.2α} and pEXP-PMCA4b) for PDZ Domain Arrays (Panomics). The same codons of LTCC were inserted into the pGEX-4T-3 (GE Healthcare Bio-Sciences AB, Uppsala) vector to produce Glutathion-S-Transferase (GST) fusion proteins (pGEX-4T-3-LTCC). The expression constructs pGEX-4T-1-nNOS-PDZ and pcDNA3-nNOS were kind gifts from David Bredt (University of California, San Francisco, CA). The plasmids pRK5-kinase-MAST205 and pRK5-kinase-PDZ-MAST205 were kind gifts from Rafael Pulido (Centro de Investigacion Principe Felipe, Valencia, Spain) and the plasmid pcDNA3-Ca\textsubscript{1.2α} was a gift from Sebastian Maier (University of Wuerzburg, Germany).

2.2. TranSignal PDZ Domain Array. Plasmid constructs pEXP-Ca\textsubscript{1.2α} and pEXP-PMCA4b were transformed into E. coli BL21 (DE3) bacteria, which were then inoculated in 3 mL of LB/Amp (100 μg/mL) and shaken for an hour at 37°C at 300 rpm. After reaching an OD<sub>600</sub> of 0.5–0.8, 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to the bacteria before further growth for 3–4 h at 37°C to induce target protein expression. Cells were collected by centrifugation (4,000 × g, 10 min 4°C), resuspended in 2 mL resuspension buffer (Panomics), and lysed with a sonicator. After clearing at 14,000 rpm for 5 min at 4°C, protein content of the supernatant was quantified via bicinchoninic acid (BCA) protein assay. The PDZ Domain Arrays were prepared according to the manufacturer’s instructions, incubated with diluted bacterial extract (5 μg/mL in blocking buffer) containing the His-tagged fusion proteins for 1–2 h at room temperature, and washed thrice with wash buffer for 5 min. They were then incubated with 1x Anti-Histidine horse radish peroxidase (HRP) conjugate (Panomics), diluted in wash buffer, for 1–2 h at room temperature. Antibody complexes were detected by enhanced chemiluminescence, using ECL Western blotting substrate (ECL Plus, GE Healthcare). X-rays were scanned and the signals were quantified with ImageJ. The values were standardized against the GST negative control.

2.3. Purification of GST Fusion Proteins. GST and GST fusion proteins were expressed in E. coli BL21(DE3) by induction with 1 mM IPTG for 6 h. Bacterial cells were pelleted, resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 100 mM Na\textsubscript{2}HPO\textsubscript{4}, 2 mM KH\textsubscript{2}PO\textsubscript{4}, and pH 7.4) containing protease inhibitor (complete EDTA-free Protease Inhibitor Cocktail Tablets, Roche), and lysed by addition of lysozyme (1 mg/mL) and sonication. The lysate was cleared by centrifugation at 30,000 g for 20 min at 4°C. The pellet was resuspended in PBS and the resulting lysate was incubated with Glutathione-Sepharose (GE Healthcare) at constant rotation for 2 h at 4°C. The quantity of the washed and bound fusion proteins was estimated by Coomassie Blue staining of SDS-polyacrylamide gels.
2.4. GST Pull-Down Assays. To prepare tissue lysates, organs were removed from mice and immediately homogenized in cold RIPA buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% Na-Deoxycholate, protease inhibitor, and optional 0.1% SDS). The homogenate was centrifuged at 4,000 x g for 3 min. For cell lysates, the same RIPA buffer was used. 500 µg of each supernatant and 3 µg of bound GST or GST fusion proteins on agarose beads were rotated overnight at 4°C. The beads were pelleted and washed three times in PBS with protease inhibitors. Bound proteins were eluted in 2x Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris HCl, and pH 6.8) [33] and separated on polyacrylamide gels followed by transfer onto nitrocellulose. Nitrocellulose membranes were blocked in TBST (TBS + 0.1% Tween) with 5% milk and incubated with primary and secondary antibodies. HRP-coupled secondary antibodies were detected using ECL Plus.

2.5. Coimmunoprecipitations. HEK 293 cells (DMEM supplemented with 10% FCS), ECV cells (DDEM supplemented with 10% FCS, 4.5 g/L glucose), and HEK 293 cells stably expressing α1b (Ca1.2b) and the Ca2.2α subunit of the smooth muscle L-type calcium channel [34, 35] (a gift from Andrea Welling, TU Munich, Germany) (DDEM supplemented with 10% FCS, 200 µg/mL G418 plus 100 µg/mL hygromycin B) were grown to ~80% confluency. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After ~48 h, cells were rinsed with PBS and lysed in RIPA buffer without SDS. Subsequent to 10 min incubation on ice, cells were scraped from the plates and pelleted at 13,000 x g for 10 min at 4°C. 300 µg of the lysate was used for each immunoprecipitation. 1–5 µg of antibodies: anti-Ca1.2 (Alomone Labs), anti-MAGI-3 (Abcam), anti-HA (Covance), and anti-PMA-C4 (Sigma Aldrich), monoclonal mouse anti-PMCA 5F10 (Sigma) and Alexa Fluor 488 goat anti-mouse, and Alexa Fluor 594 goat anti-rabbit (Invitrogen). As controls, the secondary Alexa Fluor-labelled antibodies were used alone. Confocal micrographs were obtained with an Eclipse E600 Nikon microscope using a CI confocal scanning head and a 60-fold oil immersion objective.

2.6. Antibodies for Immunoblotting. The following antibodies were used for immunoblotting: anti-ZO-1 (BD Transduction Laboratories) used at 1:1000 dilution, anti-nNOS (Zymed Laboratories) used at 1: 2000 dilution, anti-Ca1.2 (Alomone Labs) diluted 1:200, anti-CASK (BD Transduction) diluted 1:1000, anti-NHERFI (Cell Signaling) diluted 1:1000, anti-MAGI-3 (Abcam) diluted 1:1000, and anti-HA (Covance) diluted 1:1000. Secondary goat anti-mouse antibodies were purchased from Jackson Immuno Research and used at 1:5000 dilution, goat anti-rabbit (Jackson Immuno Research) used at 1:10000 dilution. From eBioscience we used rabbit IgG TrueBlot (diluted 1:1000) and mouse IgG TrueBlot (diluted 1:1000).

2.7. Immunohistochemistry. Rat hearts embedded in Tissue-Tek (Sakura) were frozen in liquid nitrogen, and cryosections (20 µm) were prepared. Cryosections were placed on glass slides, fixed in 4% paraformaldehyde/PBS for 10 min, permeabilized with 0.2% TritonX-100/PBS for 20 min, and blocked with 5% goat serum in PBS for 1 h to reduce nonspecific binding. Sections were incubated with primary antibodies overnight at 4°C, washed thrice in PBS, followed by incubation with the appropriate secondary antibodies. Stained sections were washed three times in PBS and mounted in Mowiol. The following antibodies were used: polyclonal rabbit anti-Ca1.2-ATT0 488 (Alomone Labs), polyclonal rabbit anti-NHERFI (Cell Signaling), polyclonal rabbit anti-MAGI-3 (Abcam), monoclonal mouse anti-PMCA 5F10 (Sigma) and Alexa Fluor 488 goat anti-mouse, and Alexa Fluor 594 goat anti-rabbit (Invitrogen). As controls, the secondary Alexa Fluor-labelled antibodies were used alone. Confocal micrographs were obtained with an Eclipse E600 Nikon microscope using a CI confocal scanning head and a 60-fold oil immersion objective.

2.8. Tricine-SDS-PAGE. To separate low molecular weight proteins, we used the following reagents: anode buffer (0.2 M Tris, pH 8.0), cathode buffer (0.1 M Tris, 0.1 M Tricine (Sigma Aldrich), 0.1% SDS, and pH 8.25), gel buffer (3.0 M Tris, 0.3% SDS, and pH 8.45), separating gel monomer 16.5:1 (49.5% T 6% C) and stacking gel monomer 33:1 (49.5% T 3% C), whereas T denotes the total percentage of acrylamide and bisacrylamide (Roth) and C the percentage of the crosslinker relative to the total concentration T [36]. Separating gel solution (16.5% T 6% C) was first prepared by mixing 10 mL separating gel monomer, 10 mL gel buffer, and 3.2 mL gel glycerol (Merck). For the stacking gel (4% T 3% C), we used 1 mL stacking gel monomer, 3.1 mL gel buffer, and 8.4 mL H2O. We then added 100 µL APS (Sigma Aldrich) and 10 µL TEMED (Sigma Aldrich) to both gel mixtures. After addition of cathode and anode buffer, electrophoresis was performed at 4°C at 30 V and 200 mA. After entering the stacking gel, the conditions were set to 90 V and 300 mA for 5 h. Gels were subsequently stained with Coomassie Brilliant Blue G250 (Merck).

2.9. Patch-Clamp Recordings. For patch-clamp experiments, HEK 293 cells were transiently transfected with either GFP alone or nNOS and GFP. Transfections were carried out as described above. Cells were analyzed 36 h after transfection. Membrane currents were measured in the whole-cell configuration of the patch-clamp technique. During the recordings, transfected cells were superfused by a bath solution containing NaCl 82 mM, TEA-Cl 20 mM, BaCl2 30 mM, CsCl 5.4 mM, MgCl2 1 mM, EGTA 0.1 mM, glucose 10 mM, HEPES 5 mM, pH 7.4 adjusted with NaOH; 302.9 mOsm. The perfusion chamber was mounted onto a stage of an inverted fluorescence microscope. Transfected cells were selected by GFP fluorescence. For whole-cell recording, patch pipettes of
3–5 MΩ were made from borosilicate tubes using a DMZ-
Universal Puller (Zeitz). Pipettes were filled with a pipette
solution containing CsCl 102 mM, TEA-Cl 10 mM, MgCl2
1 mM, Na2ATP 3 mM, HEPES 5 mM, pH 7.4, adjusted with CsOH; 248 mOsm. Membrane currents
were recorded using an EPC-10 computer-controlled patch-clamp
amplifier in conjunction with the software TIDA
for data acquisition and analysis. The access resistance
was compensated for values lower than 10 MΩ. For analysis
of voltage-dependent activation, steady-state currents were
plotted against the membrane potentials of the electrical
stimulation. Plots of each individual cell were fitted using the
Boltzmann equation. Statistical significance was tested using
one-way analysis of variance (ANOVA). All data were given
as mean ± SEM. n = number of independent experiments; *
= statistical significance with P < 0.05. Mean values of
data obtained from Boltzmann fits were calculated for each
individual cell.

3. Results

3.1. Expression of PDZ Array Ligands and PDZ Domain Arrays
I-IV. After comparison of C-terminal ends of mammalian
Ca1.2 and PMCA4b (Figure 1(a)), the nucleotide sequences
coding for the C-termini were cloned into pEXP bacterial
expression vectors (Figure 1(b)). Verification of expression
and size of His-tagged recombinant proteins in bacteria via
Tricine gel analysis confirmed high expression levels and
expected sizes of proteins, that is, for the pEXP read-through,
pEXP-Ca1.2x and pEXP-PMCA4b, 9 kDa, 8.47 kDa, and
8.97 kDa, as calculated, respectively (Figure 1(c)). Probing
the PDZ Domain Arrays with these bacterial lysates and
subsequent detection of interactions with anti-6xHis
antibodies revealed a series of positive spots on all PDZ
arrays tested. As an example, typical results are displayed
in Figure 2. Figure 2(a) gives an overview of the arrange-
ment of the PDZ Domain Array III, and the corresponding
results for Ca1.2 and PMCA4b are shown in Figures 2(b)
and 2(c), respectively. Subsequent ImageJ quantifications of
all four PDZ arrays tested are listed in the Supplemental
Tables 1–4 in Supplementary Material available online at
http://dx.doi.org/10.1155/2013/265182. The exemplary PDZ
Domain Array III was spotted with PDZ domains of scaf-
folding proteins, especially MAGUKs. ImageJ quantification
(Supplemental Table 1) revealed that both ligands interacted
strongly with PDZ domains of MAGI-1, MAGI-2, and MAGI-
3, and PDZ domains of SCRIBI, and TIP1.

Incubation of the PDZ Domain Array I, on which mainly
PDZ domains of synaptic proteins were spotted (overview in
Supplemental Figure I(A)), with the C-terminal PDZ ligands
of Ca1.2x and PMCA4b, revealed a panel of additional
positive PDZ spots, representing possible interaction part-
ners of the Ca1.2x and the PMCA4b (Supplemental Figures
1(B) and 1(C)). ImageJ analysis of these signal intensities
is listed in Supplemental Table 2. In this case, the Ca1.2x
and PMCA4b C-termini interacted strongly with the PDZ
domains of Mint-2-D1, OMP25, and Dlg-D1, and weakly with
CASK-PDZ. In addition to these bindings, a promiscuous
binding of the Ca1.2x C-terminus to HtrA2, hCLIMI,
hPTP1-E-D1, RIL, and ZO-2-D3 was detected. Besides the
binding of the PMCA4b C-terminus to the PDZ domains
of Mint-2-D1, OMP25, Dlg-D1, and CASK, the interaction
with the Dlg PDZ domain 2 was very prominent and the signal
strength was far above that of the positive controls.

The PDZ Domain Array II is arranged as shown in
Supplemental Figure 2(a) and includes some tight junction
proteins, sodium/hydrogen exchanger proteins, and other
PDZ domains. Strong interactions for both PDZ ligands
were observed for ZO-1-D1 and MAST-205 and for the PDZ
positive controls (SAP-102). Ca1.2x C-terminus also had a
high affinity for 4 other PDZ domains: ZO-1-D2, NHERF1-
D1, NHERF2-D1/D2, some KIAA proteins, and nNOS (Sup-
plemental Figures 2(B) and 2(C), analyses of signal intensities
summarized in Supplemental Table 3).

Probing the PDZ Domain Array IV, mainly consisting of
an assortment of scaffolding proteins, MAGUKs, Lin-
7 proteins, nucleotide exchange factors, and syntrophins
(Supplemental Figure 3(A)), revealed strong interaction of
the PMCA4b C-terminus with PDZ domains of MUPPI,
Figure 2: PDZ Domain Array III. (a) Schematic representation of the TranSignal PDZ Domain Array III. 100 ng of the PDZ domain containing proteins are spotted in duplicate on the array. Pos: positive control (Histidine-tagged ligand), negative control-Glutathione-S-Transferase (GST). (b) PDZ Domain Array III was incubated with the bacterial lysate containing the Histidine-tagged recombinant protein, pEXP-Ca\(_{1.2}\). (c) PDZ Domain Array III was incubated with the bacterial lysate containing the Histidine-tagged recombinant protein, pEXP-PMCA4b. Both bacterial extracts had a concentration of 5 \(\mu g/mL\). PDZ domain and ligand interactions were visualized with anti-Histidine antibodies.
Dlg2 (Chapsyn-110), Dlg3 (SAP-102), LIN7A, LIN7B, LIN7C, SNAI, and SNBL. Both PDZ ligands interact with the PDZ domains of GEFII, GEFI2, and SHKI, and the Ca,1.2x C-terminus binds to that of PIST (results in Supp. Figures 3(B) and 3(C) and initial quantification of dot intensities summarized in Supp. Table 4).

For further analyses, some of already established and some of newly identified interaction partners were selected (listed in Table 1), and the interactions were verified in coimmunoprecipitation and colocalization experiments.

3.2. Coimmunoprecipitations of Ca,1.2x with Putative Interaction Partners. The interaction of Ca,1.2x with different members of MAGUKs (CASK, MAGI-3, and ZO-1) and the proteins NHERF1 and MAST-205 was verified by coimmunoprecipitations. CASK, a 112 kDa protein, is expressed at neuronal synapses, where it interacts with neurexin, and in renal epithelial cells [37]. Therefore, we examined the putative interaction between Ca,1.2x and CASK in lysates of HEK 293 cells, which were transfected with Ca,1.2x. Coimmunoprecipitation of CASK with an anti-Ca,1.2x antibody was further indicative for an interaction of the proteins (Figure 3(a)).

NHERF1, also known as Ezrin binding protein 50, is a 55 kDa phosphoprotein containing two PDZ domains [38]. To test for interaction of full-length proteins, HEK 293 cells, stably expressing the α and the β subunits of Ca,1.2x, were subsequently transfected with NHERF1 and coimmunoprecipitations using lysates of these cells were performed. NHERF1 was also coprecipitated with the Ca,1.2-specific antibody in transfected cells (Figure 3(b)).

MAGI-3 (160 kDa) is predominantly expressed in brain but also in other organs [39–41]. Similar to CASK and NHERF1, coimmunoprecipitations revealed interaction of Ca,1.2x and MAGI-3 in mouse brain lysates (Figure 3(c)).

The tight junction protein ZO-1 is expressed in epithelial and, to a lesser extent, also in endothelial cells [42, 43]. Therefore, we searched for an interaction between Ca,1.2x and ZO-1 in Ca,1.2x-transfected endothelial ECV cells. As depicted in Figure 3(d), the prominent band suggested a robust interaction of Ca,1.2x and ZO-1 in ECV cells.

The serine/threonine kinase (Ser/Thr kinase) MAST-205 is expressed in testis, brain, and kidney tissues [44, 45]. To test for protein interactions between Ca,1.2x and MAST-205, we used the HA-tag constructs pRk5-kinase-MAST-205 (36 kDa) and pRk5-kinase-PDZ-MAST-205 (77 kDa), precipitated with the Ca,1.2-specific antibody, and detected the signal with a HA-specific antibody. In this case, the kinase domain alone (third lane) and also the PDZ-containing construct were coprecipitated (Figure 3(e)).

3.3. Coimmunoprecipitations of PMCA4b with Putative Interaction Partners. To confirm our data from the PDZ arrays, we tested for interaction between PMCA4b and CASK by coimmunoprecipitations from kidney and brain lysates, and transfected HEK 293 cells (Figure 4(a)). The results confirmed interaction of PMCA4b and CASK, as previously demonstrated [23]. Binding of the proteins PMCA4b and ZO-1 was demonstrated in extracts from various sources. The PMCA-specific antibody coprecipitated the 220 kDa protein ZO-1 in all cell lysates tested (Figure 4(b)). In both immunoblots, the positive controls were inputs of PMCA4b-transfected HEK 293 cells and the negative controls were the positive controls incubated with A/G-agarose and an irrelevant antibody (anti-AT,).
Figure 3: Coimmunoprecipitation of Ca\(_{\alpha1,2}\). (a) Coimmunoprecipitation demonstrating an interaction of Ca\(_{\alpha1,2}\) with CASK; CASK was expressed in HEK 293 cells, which we additionally transfected with pcDNA3-Ca\(_{\alpha1,2}\). We also probed mouse brain lysates. These lysates were precipitated with polyclonal \(\alpha\)-Ca\(_{\alpha1,2}\) antibody and probed with monoclonal \(\alpha\)-CASK antibody during immunoblotting (IB). The positive control (input) consisted of 20\(\mu\)g of the HEK 293 lysate. The negative control was HEK 293 cells immunoprecipitated (IP) with an irrelevant antibody (\(\alpha\)-NFATc2). (b) Interaction between Ca\(_{\alpha1,2}\) and NHERF1. We precipitated HEK 293 cells stably expressing NHERF1 with polyclonal \(\alpha\)-Ca\(_{\alpha1,2}\) antibody, and also tissue lysates of heart and kidney. For IB we used \(\alpha\)-NHERF1 antibody. (c) IP demonstrated an interaction of Ca\(_{\alpha1,2}\) with MAGI-3. Positive and negative controls are as described above. Ca\(_{\alpha1,2}\) antibody was used for IP and MAGI-3 antibody for IB. (d) Interaction between Ca\(_{\alpha1,2}\) and ZO-1. ZO-1 protein is expressed in ECV cells, hence the positive control was nontransfected ECV cells; the negative control was HEK 293 cells immunoprecipitated with an irrelevant antibody (\(\alpha\)-NFATc2), and for the IP we used Ca\(_{\alpha1,2}\) antibody for precipitation and \(\alpha\)-ZO-1 for the IB. (e) HEK 293 cells with stable overexpression of \(\alpha\) and \(\beta\) subunits of Ca\(_{\alpha1,2}\) transfected with HA-KD (36 kDa) or HA-KD+PDZ domain of MAST-205 (77 kDa) were incubated with \(\alpha\)-Ca\(_{\alpha1,2}\) and protein complexes were subsequently precipitated with protein A/G beads. Western blots were probed with HA antibodies. Irrelevant antibodies were used in negative controls and nonprecipitated organ lysates as positive controls.
The voltage-gated L-type calcium channel, Ca\textsubscript{1.2}, and the plasma membrane calcium ATPase, PMCA4b, play major roles in excitable and nonexcitable cells. Ca\textsubscript{1.2} regulates the calcium entry into cells upon depolarization, while PMCA4b controls cellular calcium homeostasis by calcium extrusion. Both are important functional proteins in the heart and brain, but the specific tasks and the precise mechanisms are still investigated. The present studies were initiated to understand the regulatory consequence and the physiological background of the interactions from the C-terminal ligands Ca\textsubscript{1.2} and PMCA4b with PDZ domain containing proteins. Using three independent assays (PDZ Domain Array, GST pull-down, and immunoprecipitation) and colocalization studies, we could show the interaction of a multiplicity of PDZ domain containing proteins and their ligands, Ca\textsubscript{1.2} and PMCA4b.

PDZ domain proteins regulate the traffic and targeting of proteins, to assembly of signaling complexes and networks designed for efficient and specific signal transduction [6]. Presently, some of the described interaction partners of PMCA4b belong to the family of MAGUKs [18, 23, 46] but, in addition, nNOS and NHERF2 have been identified as interacting partners of PMCA C-termini [28, 29]. The C-terminal end of the PMCA splice variant 4b (ETSV) differs from other b variants [16, 17, 28], suggesting that the C-termini determine the specificity of interactions with other proteins. We identified new PDZ protein interaction partners of PMCA4b, whereby ZO-1, MAGI-1-3, Mint-2, and MAST-205 are of primary importance. For Ca\textsubscript{1.2}, we detected the same combination of proteins, with the addition of CASK, NHERFI, NHERF2 and nNOS.

Zonula occludens proteins are regulators of tight junction (TJ) assembly, and recent investigations have shown that these proteins also promote adherens junction (AJ) assembly [47]. The protein binding between the PDZ domain 1 of ZO-1 and the C-terminus of PMCA4b is insofar likely because both proteins are located at the membrane (Figure 4(b), Supplemental Figure 2(C)). A similar interaction is observed between the C-terminus of Ca\textsubscript{1.2} and the PDZ domains 1 and 2 of ZO-1 (Figure 3(d) and Supplemental Figure 2(B)). We suggest that this protein interaction is probably important for the regulation of calcium ions and cytoskeletal dynamics at cell junctions and the plasma membrane.

In our experiments we detected that the three MAGI proteins (MAGI-1 domain 3; MAGI-2 and MAGI-3 domain 6) bind to the C-terminus of PMCA4b and Ca\textsubscript{1.2} (Figures 2(b)–2(c) and Figure 3(c)). MAGI-1 and MAGI-3 are widely expressed in tissues like brain, heart, lung, and colon, but tend to localize to tight junctions of epithelial cells [48, 49]. MAGI-2 is exclusively widespread in neuronal tissue [50]. The group of Hall [51, 52] reported that the β1 adrenergic receptor (β1AR) binds MAGI-2 and MAGI-3. MAGI-2 enhances the receptor’s association with β-Catenin and its internalization, while MAGI-3 inhibits G\textsubscript{i}-mediated ERK activation by β1AR. MAGI-2 and MAGI-3 also bind to the tumor suppressor gene product of PTEN (a tumor suppressor phosphatase). These MAGI proteins support PTEN suppression of Akt, which
Figure 5: Colocalization of \( \text{Cav1.2} \) with NHERF1 and MAGI-3, and PMCA4b with MAGI-3 in rat cardiomyocytes. Double immunofluorescent staining of \( \text{Cav1.2} \) and NHERF1 (a)–(c), MAGI-3 and \( \text{Cav1.2} \) (d)–(f), and PMCA4b and MAGI-3 (g)–(i) in rat cardiomyocytes. For heart sections the following antibodies were used: polyclonal rabbit anti-\( \text{Cav1.2} \)-ATTO488 ((a), (d)), polyclonal rabbit anti-NHERF1 (b), polyclonal rabbit anti-MAGI-3 ((e), (g)), and monoclonal mouse anti-PMCA5F10 (h) followed by Alexa Fluor 488 goat anti-mouse, or Alexa Fluor 594 goat anti-rabbit, where appropriate. (c), (f), and (i) are merged images. \( \text{Cav1.2} \) and NHERF1 are coexpressed at the intercalated discs (see arrow →), \( \text{Cav1.2} \) and MAGI-3 at the intercalated discs and transverse tubules (T-tubuli) (see arrow →). PMCA4b and MAGI-3 are located at the T-tubules as well (see arrow →).

is involved in apoptosis suppression and growth induction [41, 53]. However, MAGI-3 is localized to diverse cellular compartments including the nucleus, cytoplasm, and junctional complexes at the cell surface [54], making it a central modulator of its function as scaffold protein. It is interesting that both of our investigated ligands, PMCA4b and \( \text{Cav1.2} \), interact with all three MAGI proteins since the scaffolds are components of signaling complexes implicated in processes that require calcium. PMCA4b and \( \text{Cav1.2} \) may play key roles in the arrangement of calcium-dependent AJs, and thus be
Figure 6: Cav1.2α C-terminal end interaction with PDZ domain of nNOS. (a) Lysates from mouse organs (aorta, brain) and lysates from untransfected HEK 293 cells and HEK 293 cells transfected with pcDNA3-Cav1.2α were incubated with Glutathione-Sepharose beads containing equal amounts of GST (pGEX-4T-3) and the nNOS PDZ domain fused to GST (pGEX-4T-1-nNOS-PDZ). For detection we used α-Cav1.2 (190–210 kDa). An interaction is observed between Cav1.2α and the GST fusion protein pGEX-4T-1-nNOS-PDZ, but not with GST. (b) Lysates were the same as described above. Here the HEK 293 cells were transfected with pcDNA3-nNOS. The negative control was GST (pGEX-4T-3). For detection we used α-nNOS (160 kDa). An interaction between nNOS to the fusion protein pGEX-4T-1-LTCC, where the final C-terminal 10 amino acids were fused to GST, was shown. (c) Coimmunoprecipitation demonstrated an interaction of Cav1.2α with nNOS. HEK 293 cells were transfected with pcDNA3-nNOS. 20 μg of the protein lysate was used directly as input for SDS-polyacrylamide gel electrophoresis. The negative controls were HEK 293 cells transfected with nNOS expression constructs immunoprecipitated with an irrelevant α-rabbit antibody (α-AT2), and the last lane contained HEK 293 cells (stably expressing the α and β subunits of Cav1.2) co-transfected with pcDNA3-nNOS, and immunoprecipitated with α-Cav1.2 antibody. For detection, we used the antibody α-nNOS.

Table 1: Summary of further investigated potential interaction partners.

<table>
<thead>
<tr>
<th>Full name of protein</th>
<th>Accession</th>
<th>PDZ domain</th>
<th>Mean grey values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium/calmodulin-dependent serine protein kinase (CASK)</td>
<td>O14936</td>
<td>CASK</td>
<td>11.0 4.4</td>
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<td>Microtubule-associated testis specific serine/threonine protein kinase (MAST205)</td>
<td>Q6P0Q8</td>
<td>MAST205</td>
<td>77.7 53.5</td>
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<td>Solute carrier family 9 (sodium/hydrogen exchanger) 3 regulatory factor 1 (NHERF1), domain 1</td>
<td>O14745</td>
<td>NHERF1-D1</td>
<td>84.8 0</td>
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<tr>
<td>Tight junction protein 1 (Zonula occludens) ZO1, domain 1</td>
<td>Q07157</td>
<td>ZO1-D1</td>
<td>130.7 40.6</td>
</tr>
<tr>
<td>Nitric oxide synthase 1 (neuronal) nNOS, domain 5</td>
<td>P29475</td>
<td>nNOS</td>
<td>36.2 0</td>
</tr>
<tr>
<td>Membrane-associated guanylate kinase-related 3 (MAGI-3), domain 6</td>
<td>Q5TCQ9</td>
<td>MAGI3-D6</td>
<td>72.7 203.3</td>
</tr>
</tbody>
</table>

involved in regulation of cell growth, cell morphology, and cell differentiation.

The Mint protein family (muc18-1-interacting protein) has three members, Mint-1, Mint-2, and Mint-3 [55, 56]. The Mint family plays a role in the arrangement of multiprotein complexes, and their ability to control the signaling and trafficking of membrane proteins [57]. Mints bind to Munc-18, a protein necessary for synaptic vesicle exocytosis, and to CASK, which is involved in targeting and localization of synaptic membrane proteins [55, 58–63]. The presence of PDZ domains in Mints indicates a potential involvement of these proteins in connecting synaptic vesicles to the sites of synaptic intercellular junctions [24]. The multiprotein complex between our investigated ligands and Mint proteins could play a role in the exocytosis of synaptic vesicles, as the process requires a Ca²⁺ trigger and the resultant release of neurotransmitters is a Ca²⁺-dependent reaction (Supplemental data).

MAST-205 (microtubule-associated serine/threonine kinase) is highly expressed in testis [44] and in kidney, adrenal glands, hindbrain, small intestine, and colon tissues [45]. This protein possesses a Ser/Thr kinase and one PDZ domain. Few protein interactions with MAST-205 have been identified. For example, the PDZ domain of MAST-205 additionally binds to PTEN, which regulates the cell growth and apoptosis [64]. The phosphorylation
Figure 7: Electrophysiological properties of Ba$^{2+}$ currents from Cav1.2 subunits. (a) Pattern of electrical stimulation. The membrane potential was clamped at a holding potential of $-70$ mV. From the holding potential the cells were depolarized by nine voltage steps with $+10$ mV incremental amplitudes and 50 ms duration. (b) Ba$^{2+}$ currents induced by the electrical stimulation shown in (a) in a cell expressing wild-type Cav1.2 channels. (c) Cav1.2 channel Ba$^{2+}$ currents induced by the electrical stimulation shown in (a) in a cell expressing nNOS. (d) Maximal current density of control Cav1.2 currents, Cav1.2 subunits in the presence of nNOS. (e) Voltage dependence of Ba$^{2+}$ currents: currents were normalized to the maximal current amplitude and plotted against the potentials of the electrical stimulation; the curve was fitted using the Boltzmann equation. (f) Activation threshold of Ba$^{2+}$ currents from Cav1.2 subunits and Cav1.2 subunits in the presence of nNOS; the numbers indicate the level of significance. (g) Voltage of half-maximal activation Ba$^{2+}$ currents from Cav1.2 subunits and Cav1.2 subunits in the presence of nNOS; the $V_{1/2}$ values were significantly larger in the presence of nNOS. (h) Slope of Boltzmann curve ($k_{act}$) of Ba$^{2+}$ currents from Cav1.2 subunits and Cav1.2 subunits in the presence of nNOS; the $k_{act}$ values were significantly larger in the presence of nNOS. (i) Comparison of the voltages of maximal current amplitudes ($V_{max}$); in the presence of nNOS $V_{max}$ was shifted towards more positive voltages of currents from Cav1.2 subunits.
of PTEN by the kinase domain of MAST-205 suggests that PTEN could be a physiological substrate. The group of Yun demonstrated that MAST-205 modulates the transport activity of Na$^+$/H$^+$ exchanger (NHE3) in the renal proximal tubule, and this regulation was dependent on the presence of the kinase motif in MAST-205 [45]. Our studies suggest that the C-terminal tail of PMCA4b and Ca$_{1.2}$ may act as component for specific and efficient PDZ domain recognition, which could be important in the control of PMCA4b and Ca$_{1.2}$ protein phosphorylation, stability, and function (Figure 3(e), Supplemental Figures 2(B)-2(C)).

We identified the PDZ domain containing protein CASK as a functional interaction partner of Ca$_{1.2}$ (Supplemental Figures 1(B) and 3(A)). The MAGUK protein CASK consists of a Ca$^{2+}$-calmodulin kinase, a PDZ domain, an SH3 domain, and a guanylate kinase domain. It is mainly expressed at the neuronal presynaptic membrane, interacting with neuriligin-associated neurexin [65–67], and additionally expressed in epithelial cells [37]. Mutation or deletion of CASK results in unusual synaptic function and perinatal death in mice [65], verifying its importance for brain development and function. CASK controls synapse formation and synaptic strength, and mutation or deletion in the gene leads to mental retardation [68]. All these studies indicate that Ca$_{1.2}$ and PMCA4b in conjunction with CASK may play vital roles in the targeting of protein complexes in brain and epithelial cells, and in the modulation of synaptic transmission.

Other interesting interaction partners are the sodium-hydrogen exchanger regulatory factors, NHERF1 (also called EBP50) and NHERF2 (called E3KARP). In coexistence, they possess overlapping function as regulators of transmembrane receptors, transporters, and other proteins localized at or near the plasma membrane. The ERM (Ezrin, Radixin, Moesin, and Merlin) family of membrane cytoskeletal adapters is a crucial cellular target of NHERF [69, 70]. To regulate NHE3 signaling with CASK, NHERF1 (or NHERF2), Ezrin, and protein kinase A form a multiprotein signal complex connecting NHE3 to the actin cytoskeleton. This complex is proposed to facilitate the phosphorylation and downregulation of NHE3 [70–72], playing a crucial role in the proximal tubule, because H$^+$ is secreted into the lumen by NHE3, essentially maintaining the acid base balance of the kidney. Another important aspect is the relationship between NHERF and CFTR (cystic fibrosis transmembrane regulator). The interaction between CFTR and NHERF may explain CFTRs ability to regulate other transport proteins, including the epithelial sodium channel, the renal outer medullary potassium channel, and NHE3 [73, 74]. Demarco et al. 2002 [28] suggested that the PDZ domains of NHERF2 bind the D-(S/T)-X-L motif (X represents any amino acid) at C-termini. Therefore, with the exception of PMCA4b, which has an ETSV motif, PMCAs 1b–3b (motif ETSL) interact with NHERF2/2. We confirmed these results with the PDZ Domain Array (Supplemental Figure 2(C)). Additionally, we identified a new interaction between NHERF2/2 with Ca$_{1.2}$ (motif VSNL) in Supplemental Figure 2(B). Our findings and previous studies from other groups emphasize the importance of a terminal leucine residue for high affinity peptide interaction with NHERF [28, 75, 76]. The complex of Ca$_{1.2}$ and NHERF2/2 may provide an indirect link between the Ca$^{2+}$ channel and the actin cytoskeletal network, especially to stabilize the channel along the membrane and to allow its regulation by coassembled cAMP-dependent protein kinases. The PDZ domain 1 of NHERF1 is associated with SOCs (store operated calcium channel), Trp4, Trp5, and the phospholipases C$eta$1 and C$eta$2 [77], suggesting that NHERF can link the functions of SOCs to PLC$eta$ to organize calcium and phosphoinositide metabolism, and control cell metabolism and growth. Our new results suggest an involvement of NHERF2/2 in the regulation of Ca$^{2+}$ transport as well.

The colocalization of Ca$_{1.2}$ and NHERF at intercalated discs and of Ca$_{1.2}$ and MAGI-3 at the intercalated discs and the transverse tubules is noteworthy (Figures 5(a)–5(f)). The same applies for PMCA4b and MAGI-3 at transverse tubules (Figures 5(g)–5(i)). The intercalated discs of cardiomyocytes are crucial for pulse transmission and cell structure stabilization. The transverse tubules are invaginations of the plasma membrane of muscle cells that facilitate faster transfer of a depolarisation from the plasma membrane to the core of the cell. PMCA4b and Ca$_{1.2}$ are both expressed at the caveolae of the plasma membrane, as mentioned above.

nNOS plays a crucial role in cardiomyocytes [78], regulating excitation-contraction coupling [79, 80], $\beta$-adrenergic inotropic response [79], and the development of heart failure [81, 82]. nNOS has been localized to the sarcolemma and the sarcoplasmic reticulum (SR) [29, 83, 84]. We have established that the C-terminus of Ca$_{1.2}$ interacts with the PDZ domain of nNOS by means of a PDZ Domain Array, IP, and GST pull-down (Figures 6(a)–6(c), Supplemental Figure 2(B)). Burkard et al. presented data from nNOS overexpressing mice that showed the negative inotropic effect of myocardial nNOS, which can reduce Ca$^{2+}$ currents of Ca$_{1.2}$ in their model system [85]. Furthermore, Sears et al. postulate that nNOS regulates Ca$^{2+}$ influx by means of negative feedback, because increases in [Ca$^{2+}$]$v$ excite nNOS synthesis of NO, which, on the other hand, inhibits Ca$^{2+}$ influx [80]. We now confirmed these studies by measurements of Ca$^{2+}$ currents in stable HEK 293 cells transfected with nNOS.

The cells, which express Ca$_{\alpha,1.2}$ subunits showed Ba$^{2+}$ currents with properties of L-type Ca$^{2+}$ channels. The activation threshold and voltage of half-maximal activation are corresponding to that, which is commonly known for the cardiac subtype of L-type channels that corresponds with the Ca$_{1.2}$ subunit. The Ba$^{2+}$ currents from the cells, which stably express Ca$_{1.2}$ subunits showed no inactivation. This can be due to the fact that Ba$^{2+}$ was used instead of Ca$^{2+}$, which normally leads to loss of inactivation. Furthermore, the presence of inactivation is dependent on the coexpressed $\beta$-subunit. Upon nNOS expression only the voltage-dependent activation was changed. In the presence of nNOS the voltage-dependent activation was shifted towards more positive potentials and a change of the steepness of the curve of voltage-dependent activation resulted in shift in the voltage of maximal current amplitude while the activation threshold was unchanged. Thus, in the presence of nNOS, Ca$_{\alpha,1.2}$...
subunits can be activated by the same depolarizing voltage difference but due to the shift of the half-maximal activation the currents amplitudes were smaller. With this effect, the Ca\(^{2+}\) channel current is reduced, resulting in a smaller voltage-dependent Ca\(^{2+}\) increase. In cells which express Ca\(_{1.2}\) subunits Ca\(^{2+}\)-dependent function would be reduced. For example, smooth muscle cells expressing active nNOS would display smaller depolarization-induced contractions and would support NOS-dependent relaxation.

In summary, we showed that Ca\(_{1.2}\) interacts physically with nNOS, MAST-205-, MAGI-3-, NHERFI-, and ZO-1-PDZ domains; and PMCA4b with ZO-1-, MAST-205-, and MAGI-3-PDZ domains, all demonstrated via different assays (PDZ array, GST pull-down, and IP). The partial colocalization of Ca\(_{1.2}\) and MAGI-3, Ca\(_{1.2}\) and NHERFI, and PMCA4b and MAGI-3 in rat cardiomyocytes indicates that an interaction of these proteins is highly possible. From our results, we conclude that Ca\(_{1.2}\) and PMCA4b bind promiscuously to a variety of PDZ domains. The physiological consequence of some of these interactions remains to be investigated.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PSD-95</td>
<td>Postsynaptic density protein 95</td>
</tr>
<tr>
<td>SAP 90</td>
<td>Synapse-associated protein 90</td>
</tr>
<tr>
<td>PSD-93 (Chapsyn-110)</td>
<td>Channel-associated protein of synapse-110</td>
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<tr>
<td>SH3 domain</td>
<td>Src homology 3domain</td>
</tr>
<tr>
<td>RIL</td>
<td>Reversion-induced LIM protein</td>
</tr>
<tr>
<td>SIRPI</td>
<td>Scribble domain 1</td>
</tr>
<tr>
<td>TIPI</td>
<td>Tax interaction protein 1</td>
</tr>
<tr>
<td>OMP25</td>
<td>Mitochondrial outer membrane protein 25</td>
</tr>
<tr>
<td>MUPP</td>
<td>Multiple PDZ domain protein</td>
</tr>
<tr>
<td>hCLIM1</td>
<td>Human 36 kDa carboxyl terminal LIM domain protein</td>
</tr>
<tr>
<td>HtrA2</td>
<td>High temperature requirement protein A2</td>
</tr>
<tr>
<td>hPTP1E-D1</td>
<td>Protein tyrosine phosphatase 1E-domain 1</td>
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</table>

**Acknowledgments**

The authors thank Dr. Andrea Welling (Institute of Pharmacology and Toxicology, TU Munich) for providing the stably transfected HEK 293 cells (\(\alpha_{1b}\) and \(\beta_{2a}\) subunit of Ca\(_{1.2}\)). The authors greatly appreciate the gift of the plasmid constructs, pGEX-4T-1-nNOS-PDZ, and pcDNA3-nNOS from Dr. Bredt (University of California, San Francisco, USA) and Rafael Pulido (Centro de Investigacion Principe Felipe, Valencia, Spain) for pRK5-kinase-MAST-205 and pRK5-kinase-PDZ-MAST-205. The work was supported by the IZKF Wuerzburg, TP E-33 to KS.

**References**


