Cellular Physiology and Biochemistry

Cell Physiol Biochem 2014;34:474-490
DOI: 10.1159/000363016
Published online: July 30, 2014
Accepted: July 02, 2014

1421-9778/14/0342-0474$39.50/0

Original Paper

Evidence for the Interaction of Endophilin A3 with Endogenous $K_{ca}2.3$ Channels in PC12 Cells

Malika Janbein Mohamed Abo Quader Anselm Cornelius Hoppner Isabell Grüner Erich Wanker Stephanie Wälter Eva Küppers Stephan Grissmer Heike Jäger

Institute of Applied Physiology, University of Ulm, Ulm, Germany

Key Words
$K_{Ca}2.3$ channel • Endophilin A3 • Protein-protein-interaction • Yeast two-hybrid experiments • Pull-down assays • Electrophysiology • Ca^{2+} Measurements

Abstract
Background/Aims: Small-conductance calcium-activated (SK) channels play an important role by controlling the after-hyperpolarization of excitable cells. The level of expression and density of these channels is an essential factor for controlling different cellular functions. Several studies showed a co-localization of $K_{Ca}2.3$ channels and Endophilin A3 in different tissues. Endophilin A3 belongs to a family of BAR- and SH3 domain containing proteins that bind to dynamin and are involved in the process of vesicle scission in clathrin-mediated endocytosis. ***Methods:*** Using the yeast two-hybrid system and the GST pull down assay we demonstrated that Endophilin A3 interacts with the N-terminal part of $K_{Ca}2.3$ channels. In addition, we studied the impact of this interaction on channel activity by patch clamp measurements in PC12 cells expressing endogenous $K_{Ca}2.3$ channels. $K_{Ca}2.3$ currents were activated by using pipette solutions containing 1 µM free Ca^{2+}. ***Results:*** Whole-cell measurements of PC12 cells transfected with Endophilin A3 showed a reduction of $K_{Ca}2.3$ specific Cs^{+} currents indicating that the interaction of Endophilin A3 with $K_{Ca}2.3$ channels also occurs in mammalian cells and that this interaction has functional consequences for current flowing through $K_{Ca}2.3$ channels. Since $K_{Ca}2.3$ specific currents could be increased in PC12 cells transfected with Endophilin A3 with DC-EBIO (30 µM), a known SK-channel activator, these data also implicate that Endophilin A3 did not significantly remove $K_{Ca}2.3$ channels from the membrane but changed the sensitivity of the channels to Ca^{2+} which could be overcome by DC-EBIO. ***Conclusion:*** This interaction seems to be important for the function of $K_{Ca}2.3$ channels and might therefore play a significant role in situations where channel activation is pivotal for cellular function.

M. Janbein, M. Abo Quader, A.C. Hoppner and I. Grüner contributed equally.

Prof. Dr. Stephan Grissmer
Institute of Applied Physiology, University of Ulm,
Albert-Einstein-Allee 11, D-89081 Ulm (Germany)
E-Mail stephan.grissmer@uni-ulm.de

Introduction

Small-conductance calcium-activated potassium (SK) channels, a subfamily of the calcium-activated potassium channel family, are activated solely by rising of the intracellular calcium-concentration $[Ca^{2+}]_i$ in a voltage-independent manner [1]. They are widely distributed in different tissues including the brain, neurons, skeletal and smooth muscle [2-15]. SK channels play different physiological roles. For example they mediate the fast after-hyperpolarization (fAHP) following an action potential in neurons [16] and they control the hormone secretion in endocrine cells. In neurons, SK channels are not uniformly distributed but highly concentrated in neuronal dendrites [17] where they regulate the synaptic plasticity and the dendritic excitability [18]. Three different subunits of SK channels have been cloned and identified as K_{Ca}2.1, K_{Ca}2.2, and K_{Ca}2.3 (SK1, SK2 and SK3). Each subunit is made up by six transmembrane domains with intracellular NH_2- and COOH-termini [3]. These subunits differ mainly in their NH_2- and COOH-terminal domains [3]. Functional channels are stable complexes of the ion pore-forming subunits and calmodulin (CaM), which binds constitutively to the calmodulin-binding domain (CaMBD) in the COOH-terminus of each subunit [19] mediating the gating and activation of these channels by calcium [20]. It seems that different stoichiometries between the channel and CaM are important for gating [21]. The functional channels can also bind protein kinase CK2 (CK2) and protein phosphatase 2A (PP2A) with counterbalancing effects as has been shown for K_{Ca}2.2 [22, 23]. For example CK2 phosphorylates CaM in complex with the K_{Ca}2.2 channel thereby reducing the Ca^{2+}-sensitivity of the channel [22, 23].

The level of expression and the density of K_{Ca}2.3 channels is an important factor for controlling different functions, e.g. controlling the contraction of myometrial smooth muscles since the level of expression of K_{Ca}2.3 in the myometrium is not the same during all stages of pregnancy [24]. Little is known about the mechanisms controlling the expression level of SK channels and which proteins are involved.

Similar to the NH_2-terminus of the Huntingtin protein (Htt), the NH_2-terminus of K_{Ca}2.3 has 2 polyglutamine (polyQ) domains separated by several PXXP motifs (see also Fig. 1) which are known to bind to SH3 domains [25]. Sittler et al. [26] showed an interaction between Endophilin A3 and the Huntingtin protein (Htt), which is mutated in Huntington disease. That interaction is mediated by the SH3 domain of Endophilin A3 and the proline-rich region (PRR) of Htt which directly follows the polyQ domain in the N-terminal region of Htt [27]. Li and Li [28] showed that the length of the polyQ domain of Htt affects its interaction with Endophilin A3 since that interaction occurs only with Htt with an expended polyQ domain. Endophilin A3 is a member of a larger family of three Endophilin proteins that is preferentially expressed in the brain and testis [29]. Based on this data and the similarities between Htt and the N-terminal part of K_{Ca}2.3 (see also Fig. 1), we wanted to examine whether K_{Ca}2.3 channels can interact with Endophilin A3 and whether the interaction influences K_{Ca}2.3 channel activity.

We initially investigated whether the N-terminal part of K_{Ca}2.3 interacts with Endophilin A3 by means of a LexA based yeast two-hybrid system and a pull-down assay. In both experiments we found indications for an interaction of K_{Ca}2.3 with Endophilin A3. In mouse hippocampal slices we found K_{Ca}2.3 channels colocalized with Endophilin A3 indicating close proximity of the two proteins *in vivo*. We then focused on the investigation of a possible functional role of this interaction on K_{Ca}2.3 in mammalian cells. Therefore, we expressed Endophilin A3 in PC12 cells which contain endogenous K_{Ca}2.3 channels. We could demonstrate that PC12 cells transfected with Endophilin A3 showed a reduction of K_{Ca}2.3 specific Cs^+ currents indicating that the interaction of Endophilin A3 with K_{Ca}2.3 channels also occurs in mammalian cells and that this interaction has functional consequences for current flowing through K_{Ca}2.3 channels.

Materials and Methods

Plasmids / Cloning

The pcDNA3SK3N$_{(1-274),\ Q14}$ (K_{Ca}2.3 N-terminal region with 14Q), and the pcDNA3SK3N$_{(1-299),\ Q19}$ (K_{Ca}2.3 N-terminal region with 19Q) vectors coding for the N-terminal region of the K_{Ca}2.3 channel were generated as described in [30].

pGAD10-Endophilin A3, pTL1-HA-SH3GL3 were constructed as described in [26]. As control vectors we used pDNA-BD-SIM; pAD-ARNT as positive controls on selection plates as described previously [31, 32]. For the construction of pDNA-BD-SK3N$_{(1-299),\ Q19}$; pDNA-BD-SK3N$_{(1-274),\ Q14,}$ EcoRI XhoI fragments from pLexA SK3N$_{(1-299),\ Q19}$ and pLexA SK3N$_{(1-274),\ Q14}$ [30] were ligated into an EcoRI SalI digested pBTM116 vector [33] generating LexA fusionprotein with the N-terminal part of the K_{Ca}2.3 channel with 19 respectively 14 glutamine repeats.

Plasmids for pull-down experiments

The 897 nucleotides encoding the N-terminal fragment of K_{Ca}2.3 were cloned into the vector pGEX-6P1 (Amersham Pharmacia Biotech Limited, Little Chalfont, UK) using EcoRI XhoI SK3 N-tail coding fragment from pLexA SK3N$_{(1-299)}$ in fusion to Glutathione-S-transferase (GST) of pGEX-6P1. To clone a 1065 nucleotide long Endophilin A3 gene into pET100/D-TOPO (invitrogen, Carlsbad, USA), PCR-primers 5'- CACCATGTCGGTGGCCGGGCTGAAGAAG-3'and 5'-TTACTGAGGTAAAGGC-3' were used, to clone a 853 nucleotide long Endophilin A3 lacking the SH3-domain, primers 5'- CACCATGTCGGTGGCCGGGCTGAAGAAG-3' and 5'-TTAGTCTAATGCTGCCTCTATGAACACA-3' were used, respectively. After sequencing the correct PCR-product was cloned into the pET100/D-TOPO-vector and fused to a polyHis-tag.

Plasmids for Endophilin A3 expression in mammalian cells

An SH3GL3 gene (Endophilin A3) containing plasmid (pTL1-HA-SH3GL3) was used for the expression of Endophilin A3 in PC12 cells [26].

Immunocytochemistry

The following primary antibodies were used: K_{Ca}2.3 (1:500, rabbit polyclonal, alamone labs, Jerusalem, Israel) and Endophilin A3 (1:50, goat polyclonal, Santa Cruz Biotechnology, Heidelberg, Germany).

Immunocytochemistry was performed as described earlier [34]. Briefly, frozen sections were washed in 10 mM TBS (tris-buffered saline, pH 7.4). The sections were incubated in pre-incubation buffer (10 % normal swine serum (PAA Laboratories, Pasching, Germany), 0.3 % Triton X-100 (Sigma), 1 % bovine serum albumin (Sigma) in 1 x TBS for 60 min at RT, before the primary antibody diluted in antibody dilution buffer (10 % normal swine serum, 1 % BSA in 1xTBS) was added and incubated overnight at 4°C. The next day, sections were washed with TBS, and incubated with the appropriate secondary antibody (donkey-anti-goat Alexa Fluor 488nm, 1:1000; donkey-anti-rabbit Alexa Fluor 546 nm, 1:1000) in 4 % normal swine serum, 1 % BSA in 1xTBS for 60 min at RT. Subsequently, the sections were washed in TBS and sections were incubated in 1 µg/ml DAPI (4',6 Diamidino-2-phenylindole dihydrochloride, Sigma) in TBS for nuclear staining. The sections were washed in distilled water and mounted in Moviol (Sigma). To exclude unspecific staining, negative controls were performed omitting the first antibody. Fluorescence was detected using a Zeiss Imager.M2 Apoptome.

Cell culture

Cells were cultured with MEM medium containing glutamax-I and Earle`s salts (Invitrogen, Karlsruhe, Germany) and 10 % HS/5 % FCS for rat pheochromocytoma PC12 cells [30, 35]. PC12 cells endogenously express the rat version of the K_{Ca}2.3 channel (KCNN3_RAT, UniProtKB P70605 with Q19). For electrophysiological control experiments PC12 cells were transfected with 2.5 µg pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA) conferring GFP expression. For the expression of Endophilin A3, PC12 cells were transfected with a mixture of 2.4 µg pTL1-HA-SH3GL3 (pEndophilin A3) and 0.1 µg of pEGFP-N1. PC12 cells were transfected using the LipofectamineTM 2000 Transfection reagent (Invitrogen, Darmstadt, Germany) according to the manufacturers` protocol. On the second day after transfection cells were suspended and transferred to poly-lysine coated glass cover slips for the electrophysiological measurements.

Yeast two-hybrid experiments

Yeast two-hybrid experiments were performed as described earlier [30]. Briefly, yeast cultures were grown under standard conditions in liquid or solid medium with YPD or SD (minimal synthetic dropout) medium (Clontech, Heidelberg, Germany) with DO supplements (Clontech, Heidelberg, Germany) according to the auxotrophies of the yeast strains. Yeast transformations were performed using a standard protocol with TE/lithium acetate [36]. The yeast reporter strain L40c [MATα, *his3, trp1, ura3,lexA*$_{op(x8),}$ *lys2,lexA*$_{op(x4)}$ – *HIS3*] [37] was transformed with the reporter plasmid p8op*lacZ* (*lexA*$_{op(x8)}$*ura3*Amp^r) (Clontech, Heidelberg, Germany) and subsequently transformed with pBTM116 derived bait plasmids and a pGAD10 derived prey plasmid. pBTM-SIM and pARNT were used as positive controls [38, 39]. Transformants were tested for activation of reporter genes by growth on SD-trp-leu minimal plates with and without histidin and supplemented with 2 % wt/vol glucose or 2 % wt/vol galactose and 1 % wt/vol raffinose, and with 20 mg/ml X-Gal (AppliChem, Darmstadt, Germany). Selection of positive interactions was done by yeast cell growth on plates lacking Trp, Leu, His and was judged by the development of blue color (beta-galactosidase activity).

Pull-down experiments

The K_{Ca}2.3 N-terminal fragment and Endophilin A3 coding constructs were transformed into BL21 DE3 [40] cells. Protein expression was induced with 0.1 mM IPTG for 3h before lysis. The BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA) was used to determine the protein concentration of the lysates. For the pull-down-assay protein was immobilized on Cobalt chelate gel columns (ProFound Pull-Down PolyHisProtein/Protein Interaction Kit, Pierce, Rockford, Illinois, USA), and pull-down experiments were performed according to the manufacturers protocol. The immunoblots were done as described previously [30]. Primary antibodies were diluted as recommended by the manufacturer. The polyclonal anti-Endophilin A3 antibody was diluted 1:200.

Patch-clamp experiments

Patch-clamp experiments were done with the whole cell recording mode of the patch clamp technique as described previously [30, 41, 42]. For the measurements in Figures 5, 6 and 8 the internal pipette solution contained (in mM): 145 K^+ aspartate, 2 $MgCl_2$, 10 HEPES, 10 K_2EGTA, and 8.6 $CaCl_2$ (pH 7.2; 300-320 mosM) corresponding to 1 µM free Ca^{2+}-concentration. The Na^+ aspartate solution contained (mM): 160 Na^+ aspartate, 4.5 K^+ aspartate, 2 $CaCl_2$, 1 $MgCl_2$ and 5 HEPES, pH 7.4. PC12 cells contain endogenous K_{IR} channels. Since Cs^+ was described to block K_{IR} channels [43], and K_{Ca}2.3 can carry a significant Cs^+ current [44], K_{Ca}2.3 conductance was measured in Cs^+ aspartate solution. The Cs^+ aspartate solution contained (mM): 165 Cs^+ aspartate, 2 $CaCl_2$, 1 $MgCl_2$ and 5 HEPES, pH 7.4. DC-EBIO (Tocris Bioscience, Bristol, UK) was dissolved in DMSO and supplied as dilutions in Cs^+ aspartate solution with 10, 30, 100, 300, and 1000 µM DC-EBIO. To our knowledge, PC12 cells do not endogenously express K_{Ca}3.1 channels that might also be activated by DC-EBIO. In addition, current through other channels besides K_{Ca}2.3 channels would be minimal using Cs^+ as current carrier.

The Ca^{2+} measurements were done in the whole-cell mode of the patch-clamp technique as described previously [41, 42], combined with fura-2 (Invitrogen, Karlsruhe, Germany) measurements [30]. For the Ca^{2+} measurements pipettes were filled with 2 to 4 µl of tip solution (containing 50 µM fura-2, 135 mM potassium aspartate, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, pH 7.2), which was overlaid with pipette-solution (50 µM fura-2, 135 mM potassium aspartate, 2 mM $MgCl_2$, 10 mM HEPES, 1 mM EGTA, and 0.95 mM $CaCl_2$ corresponding to 3 µM free Ca^{2+}, pH 7.2). All values presented here are mean ± SD with n the number of independent observations.

Measuring intracellular Ca^{2+}

The intracellular Ca^{2+} concentration was determined as described before [30]. Briefly, for visualization, cells were placed on the stage of an Axiovert 100 microscope equipped with a Zeiss 40 X Neofluar 1.30 oil objective. The $[Ca^{2+}]_i$ was measured with the videoprobe Ca^{2+} imaging system (ETM Systems, Irvine, CA). Light from a 75-W xenon arc lamp was passed alternatively through excitation bandpass filters of 350 or 380 nm, which were exchanged by a computer controlled Lambda-10 filter wheel unit (Sutter Instruments, Novato, CA). A Hamamatsu C2400 camera obtained light from a 400 nm dichroic mirror and 480 nm long-pass emission filter. Background-corrected 350/380 ratio images were collected every 5 s. $[Ca^{2+}]_i$ was determined from the relationship $[Ca^{2+}]_i = K_{eff}$ x (R - R_{min}) / (R_{max} -R), where R is the F_{350}/F_{380} ratio, R_{min}

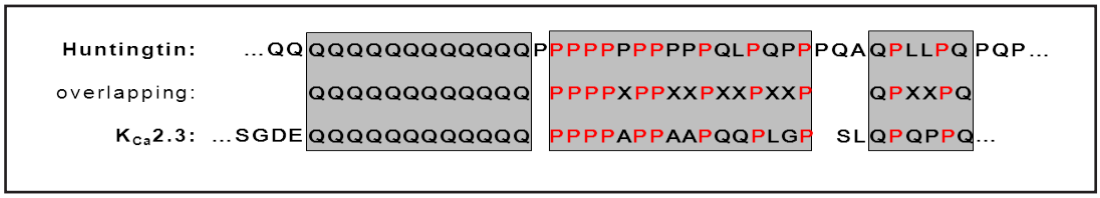

Fig. 1. Sequence comparison of Huntingtin and the N-terminal part of K_{Ca}2.3. The grey boxes indicate regions with high homology. Highlighted in red are homologous proline motives.

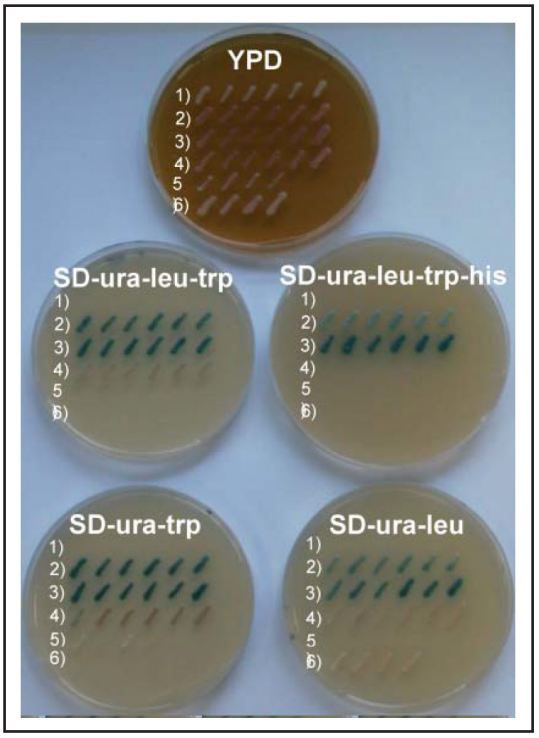

Fig. 2. Interaction of the N-terminal part of K_{Ca}2.3 with Endophilin A3. Of each transformation 4 - 6 individual colonies were streaked on SD/-Ura/-Leu/-Trp/BU-salts-, SD/-Ura/-Leu/-Trp/-His/BU-salts-, SD/-Ura/-Trp/BU-salts-, SD/-Ura/-Leu/BU-salts-minimal plates and on YPD plates as growth control. All plates, with the exception of the YPD-plate contain X-Gal as substrate for β-galactosidase. Row 1: untransformed yeast strain L40; Row 2: DNA-BD-SIM; AD-ARNT (positive control); Row 3: DNA-BD- $K_{Ca}2.3N_{(1\text{-}299),\ Q19}$; AD-Endophilin A3; Row 4: DNA-BD- $K_{Ca}2.3N_{(1\text{-}274),\ Q14}$; AD- Endophilin A3; Row 5: DNA-BD- $K_{Ca}2.3N_{(1\text{-}299),\ Q19}$; AD: - ; Row 6: DNA-BD: - ; AD-Endophilin A3. AD: activation domain, DNA-BD: DNA-binding domain.

and R_{max} are the ratios at minimal [Ca^{2+}] and saturating [Ca^{2+}] (3 μM), respectively, and K_{eff} is the effective dissociation constant. For calibration, cells were perfused with internal solution containing minimal, saturating, and 0.5 μM [Ca^{2+}] and 50 μM fura-2 (Molecular Probes, Eugene, OR). Values for K_{eff}, R_{min}, and R_{max} were calculated to be 1.134 μM, 0.1, and 1.74.

Results

The N-terminal part of the K_{Ca}2.3 channel is unique within the K_{Ca} channel family. It is about two times longer compared to K_{Ca}2.1 and K_{Ca}2.2 channels and has a high similarity to Huntingtin (Fig. 1). Since Huntingtin was shown to interact with Endophilin A3 [26] we wanted to find out whether Endophilin A3 can also interact with the N-terminal part of K_{Ca}2.3. Initially, we investigated this possible interaction using a LexA-based yeast two-hybrid system.

Yeast two-hybrid experiments

In yeast we observed an interaction between Endophilin A3 and the N-terminal part of K_{Ca}2.3 as shown in Fig. 2. As expected L40c yeast in row 1 can only grow on YPD plates. Yeast colonies in row 3 show growth and blue color on SD-ura-leu-trp-his plate indicating transcription of both reporter genes (*lacZ* and *HIS3*) and therefore a protein-protein interaction of $K_{Ca}2.3N_{(1\text{-}299),\ Q19}$ and Endophilin A3. The positive control in row 2 showed the identical phenotype. Row 5 and 6 show the result of transforming yeast with either pBTM116-

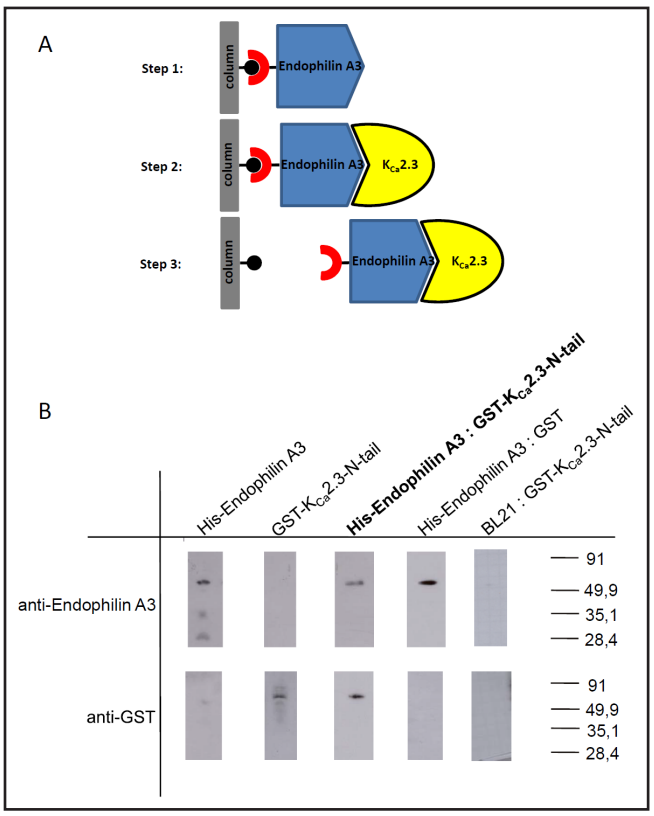

Fig. 3. Pull-down experiments. (*A*), principle scheme of the pull-down experiments. Step 1: Binding of the protein of interest (Endophilin A3) to a column. Step 2: Binding of the second protein of interest (GST-coupled N-terminal part of K_{Ca}2.3) to the first one. Step 3: Eluation of both proteins of interest. (*B*), Western blot of His-coupled Endophilin A3. First, Endophilin A3 was bound to an immobilized cobalt-chelate column and non-bound protein was removed. Second, incubation of the column-bound Endophilin A3 with the GST-coupled N-terminal part of K_{Ca}2.3, unbound protein removed by washing. Third, eluation of Endophilin A3 from the column, probes identified by gel electrophoreses and Western blotting. Upper row: anti-Endophilin A3 as primary antibody. Bottom row: anti-GST as primary antibody.

$K_{Ca}2.3N_{(1\text{-}299),\,Q19}$ or with pGAD10-Endophilin 3 only. This demonstrated that these constructs were not able to induce the reporter genes by themselves. Interestingly, the slightly shorter $K_{Ca}2.3N_{(1\text{-}274),\,Q14}$ did not interact with Endophilin A3 (row 4).

Other SH3 domain containing proteins, Abl, Lck, Fyn, Grb2, PI3KP58 did not interact with $K_{Ca}2.3N_{(1\text{-}299),\,Q19}$ in yeast (data not shown).

Taken together, these experiments showed that the N-terminal part of K_{Ca}2.3 interacted with Endophilin A3 in yeast. To test whether this interaction can also take place independent of yeast, we performed pull-down experiments.

Pull-down experiments

Pull-down experiments were also performed to test for the interaction of Endophilin A3 with the K_{Ca}2.3 channel. The principle of this method is shown in Fig. 3A. The "protein of interest", here His-tagged Endophilin A3, was bound to an immobilized cobalt chelate column (step 1), the second "protein of interest", here the GST-tagged N-terminal part of K_{Ca}2.3, was incubated (step 2) and then Endophilin A3 was eluted from the column (step 3). When both proteins interact with each other, they should be detected in the eluate. This was the case as can be seen by Western blotting in Fig. 3B: Endophilin A3 was detected with anti-Endophilin A3 as primary antibody (*top, middle*) together with the GST-tagged N-terminal fragment of K_{Ca}2.3 using anti-GST as primary antibody (*bottom, middle*). Since both interaction partners could be detected in the eluate, this was further proof for the interaction of Endophilin A3 and the N-terminal part of K_{Ca}2.3. A deletion of the SH3-domain in Endophilin A3 resulted in a lack of interaction with the N-terminal part of K_{Ca}2.3 (data not shown).

Is Endophilin A3 close to K_{Ca}2.3 in mammalian cells in vivo to consider a possible interaction?

A necessary prerequisite for a possible interaction of two proteins within a cell is the close proximity of the proteins in question. To investigate whether Endophilin A3 and K_{Ca}2.3

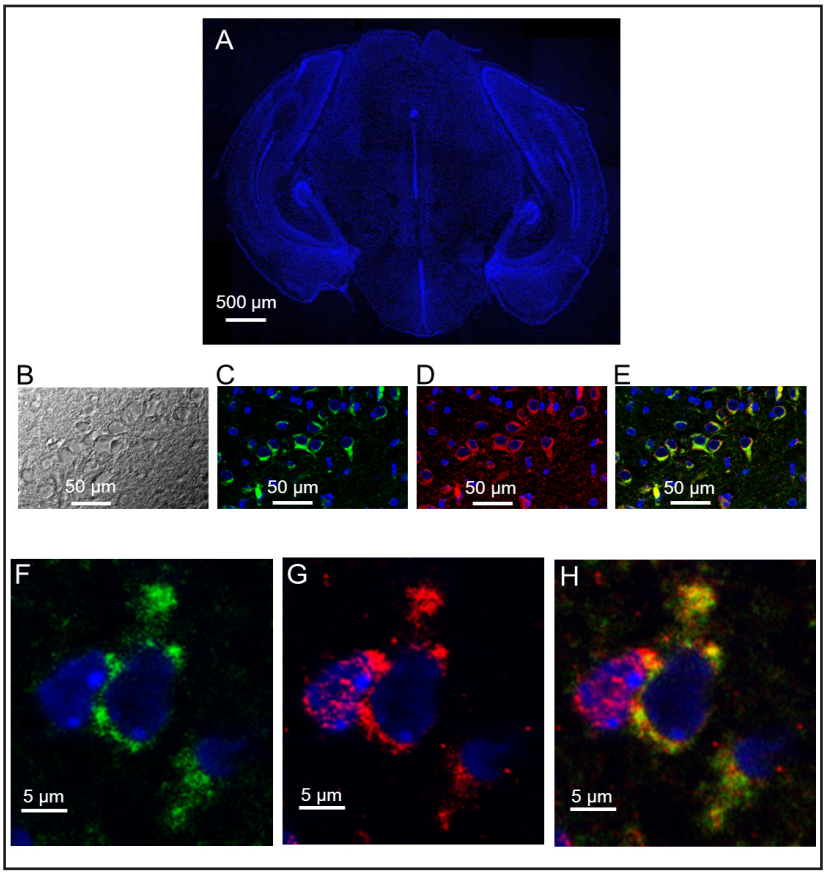

Fig. 4. Immunohistochemical costaining of K_{Ca}2.3 channels with Endophilin A3 in mouse brain slices. (*A*) Overview of a coronal slice through a postnatal (P3) mouse brain corresponding Bregma -3.6 mm; nuclei are depicted in blue (DAPI). (*B-E*) Higher magnification of the dentate gyrus in *A* using DIC (*B*), immunofluorescence for Endophilin A3 in green (*C*), K_{Ca}2.3 in red (*D*), and merged image of *C* and *D* (*E*). (*F-H*) Additional fluorescence images similar to *C-E* at higher magnification, Endophilin A3 in green (*F*), K_{Ca}2.3 in red (*G*), and merged image of *F* and *G* (*H*). Nuclei are visualized by DAPI staining (blue). Scale bars as indicated.

channels are in close proximity in mammalian cells to allow for such a possible interaction we performed immunofluorescence double-labelling studies of coronal slices of mouse brain. The result of these studies is shown in Fig. 4. This figure shows in Fig. 4A an overview of a DAPI stained slice of a mouse (P3) hippocampus (Bregma -3.63 mm). A differential interference contrast (DIC) image of a section in the dentate gyrus is shown in Fig. 4B. Immuno-labelling of Endophilin A3 (green) is shown in Fig. 4C and of K_{Ca}2.3 (red) in Fig. 4D. A merged image of Fig. 4C and 4D shows that Endophilin A3 and K_{Ca}2.3 channels co-localize in most of the cells (Fig. 4E). In addition to cells coexpressing Endophilin A3 and K_{Ca}2.3 channels, we also found cells expressing only Endophilin A3 or K_{Ca}2.3 channels as can be seen in Fig. 4F-H.

Does Endophilin A3 functionally interact with K_{Ca}2.3 in mammalian cells?

To investigate whether Endophilin A3 and K_{Ca}2.3 can functionally interact in mammalian cells, we transfected PC12 cells with pEGFP-N1 as a negative control to exclude a non-specific effect of the vector on the expression of endogenous K_{Ca}2.3 channel. First of all, we examined by whole cell measurements whether Endophilin A3 can interact with K_{Ca}2.3 channels and reduce the number of functional K_{Ca}2.3 channels in the membrane. Fig. 5 (*left*) shows a representative K_{Ca}2.3 current-amplitude of control transfected PC12 cells. At -80 mV current amplitude in Na^+ solution was -19 pA and increased in Cs^+ solution to -250 pA (Fig. 5, *left*). PC12 cells transfected with Endophilin A3 showed in Na^+ solution at -80 mV a current-amplitude of -10 pA, which did hardly increase in Cs^+ solution (Fig. 5, *middle*), indicating little activation of current through K_{Ca}2.3 channels. One possibility to explain this reduction in K_{Ca}2.3-specific current in cells transfected with Endophilin A3 could be that Endophilin A3 removed K_{Ca}2.3 channels from the membrane. This hypothesis, however, was falsified by the experiment shown in Fig. 5 (*left*). Application of a Cs^+ solution containing 30 µM DC-EBIO in the Endophilin A3 transfected PC12 cells resulted in an increase in K_{Ca}2.3-specific current to

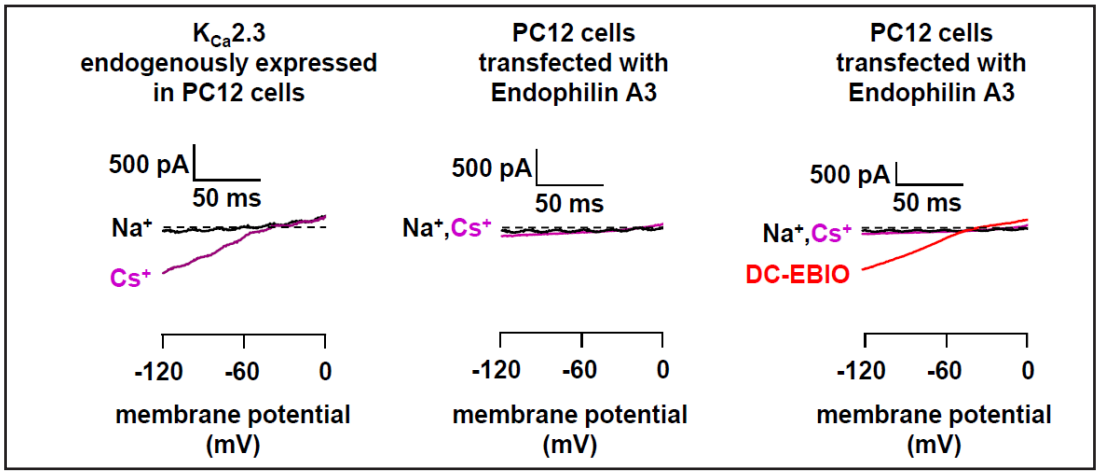

Fig. 5. Currents through endogenously expressed K_{Ca}2.3 channels in PC12 cells (*left*), after transfection with Endophilin A3 (*middle*), and after transfection with Endophilin A3 in the presence of 30 µM DC-EBIO (*right*). K_{Ca}2.3 channels were activated by pipette solutions containing 1 µM free Ca^{2+}. Whole-cell currents were elicited with 200 ms voltage ramps from -120 to +60 mV in the presence of 160 mM extracellular Na^+-solution (Na^+) and 160 mM extracellular Cs^+-solution (Cs^+). For better visualization only the voltage range between -120 to 0 mV is shown.

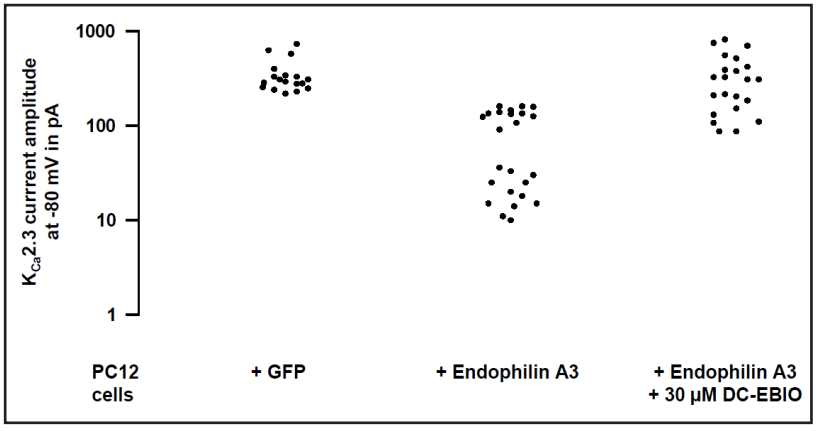

Fig. 6. Scatter plots of specific K_{Ca}2.3 current amplitudes in Cs^+ solution at -80 mV in PC12 cells from the experiments shown in Fig. 4 and similar experiments. Control transfected (GFP) PC12 cells (*left*); Endophilin A3 transfected PC12 cells without (*middle*) and with 30 µM DC-EBIO (*right*).

a value close to that obtained in control transfected cells with Cs^+ solution alone (compare Fig. 5, *left* with *right*).

The scatter plot in Fig. 6 summarizes the obtained data. K_{Ca}2.3 current amplitudes of control transfected PC12 cells at -80 mV were -350 ± 150 pA (mean ± SD, n=18), whereas current amplitudes in PC12 cells transfected with Endophilin A3 were found to be a lot smaller compared to control transfected PC12 cells. On closer look, one could even imagine that there might be two populations of Endophilin A3 transfected PC12 cells, one showing a marked K_{Ca}2.3 current decrease whereas the other is close to GFP-transfected controls, although the high current demonstrating Endophilin-transfected PC12 cells still had less current compared to the GFP-transfected cells. We would have no explanation for such heterogeneity and therefore treated the Endophilin-transfected cells as a single population with current values of -80 ± 60 pA (n=24).

The current amplitudes of PC12 cells transfected with Endophilin A3 in the presence of 30 µM DC-EBIO were comparable to those obtained in control transfected PC12 cells in Cs^+-solution without DC-EBIO (-360 ± 270 pA, n=24). Therefore, Endophilin A3 reduced the number of endogenous K_{Ca}2.3 channels functional in the membrane of PC12 cells under our

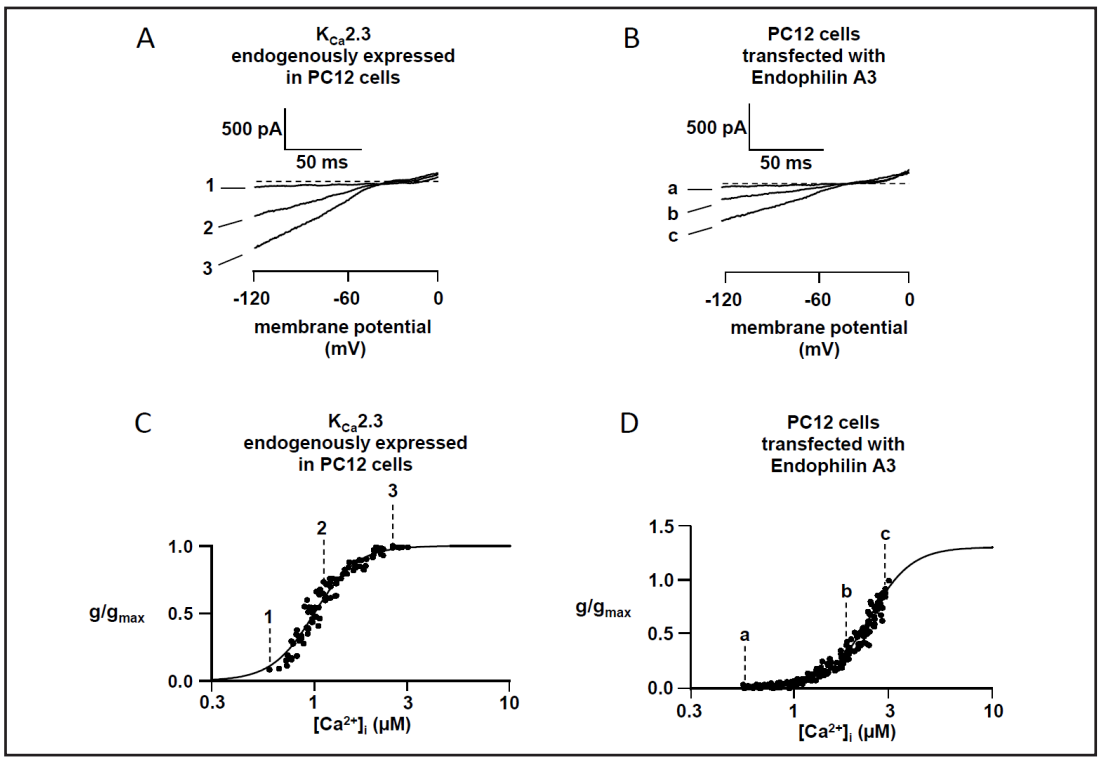

Fig. 7. Activation of K_{Ca}2.3 channels in PC12 cells control transfected (GFP) or transfected with Endophilin A3 by internal Ca^{2+}. Membrane potentials were ramped from -120 to +60 mV in 200 ms. In parallel, the internal Ca^{2+} concentration was measured by the fura-2 method. Cs^+ aspartate solution was used as external solution. Pipettes were filled with 2 to 4 µl of tip solution containing 10 mM EGTA without any Ca^{2+}, which was overlaid with pipette-solution containing 3 µM free Ca^{2+} and 1 mM EGTA (internal solutions contained 50 µM fura-2). (*A*), Representative endogenous K_{Ca}2.3 current in control transfected cells. (*B*), Representative endogenous K_{Ca}2.3 current in cells transfected with Endophilin A3. (*C*), and (*D*), for calculation of the EC_{50} values for Ca^{2+}, whole-cell conductances were determined as the slope of the ramp currents between -100 and -50 mV, were normalized to the largest slope of the ramp current observed during the experiment (g_{max}) and were plotted against the Ca^{2+} concentration measured at the same time. Concentration-response curves were fitted by eye according to the equation $g/g_{max} = A/(1+(EC_{50}/[Ca^{2+}])^{nH})$, where g_{max} is the maximal conductance observed during the experiment, EC_{50} is the half-maximal activating Ca^{2+} concentration, and n_H is the Hill coefficient (Fig. 6, *bottom*). *A* was determined by the fit and was 1.0 for C and 1.3 for D. The EC_{50} and the Hill coefficient were calculated for control (GFP) and Endophilin A3 transfected cells as mean of three independent experiments. In control transfected cells the EC_{50} was 1 ± 0.1 µM and the Hill coefficient 4.6 ± 0.2. For Endophilin A3 transfected cells the EC_{50} was 2.3 ± 0.04 µM and the Hill coefficient 5.6 ± 1.5.

measuring conditions. What was the reason for the K_{Ca}2.3 current reduction in PC12 cells transfected with Endophilin A3 since Endophilin A3 did not remove K_{Ca}2.3 channels from the membrane?

Does the interaction of Endophilin A3 with K_{Ca}2.3 channels change the Ca^{2+} sensitivity of K_{Ca}2.3 channels?

To answer that question, we examined the Ca^{2+} sensitivity of the K_{Ca}2.3 channels in control transfected and Endophilin A3 transfected PC12 cells. To do so, we measured simultaneously the whole-cell K_{Ca}2.3 conductance (Fig. 7, *top*) and the internal Ca^{2+} concentration in the same cell. This approach has the advantage over inside-out patch measurements that we can rapidly correlate current amplitude with Ca^{2+} concentration without the need for solution changes and single channel amplitude measurements. After subtraction of the intensity of the auto-fluorescent light, ratios for F_{350} and F_{380} of the fura-2 measurements were used

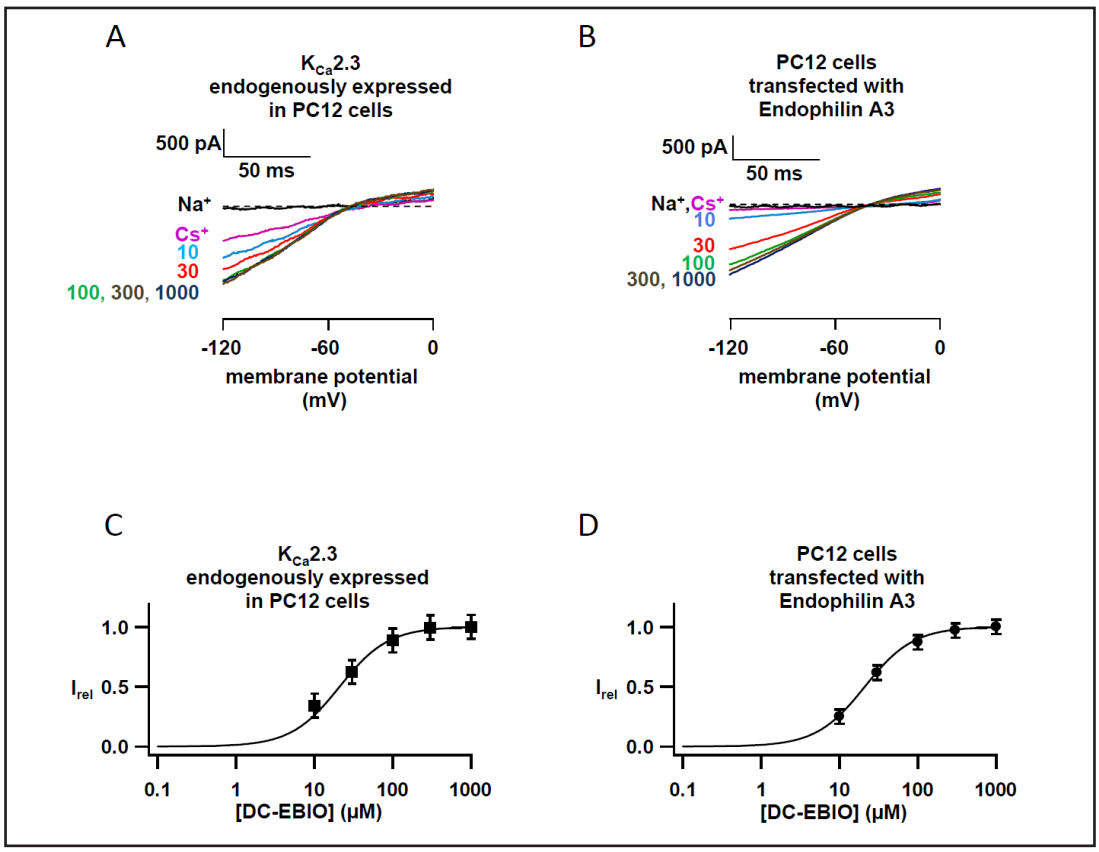

Fig. 8. Activation of endogenous K_{Ca}2.3 currents with different DC-EBIO concentrations. (*A*) representative endogenous K_{Ca}2.3 currents in control (GFP) transfected cells. (*B*) representative endogenous K_{Ca}2.3 currents in cells transfected with Endophilin A3. (*C,D*) Concentration-response curves for the activation of K_{Ca}2.3 current by different DC-EBIO concentrations (in comparison to the activation of K_{Ca}2.3 current by 1000 µM DC-EBIO) of control-GFP (*C*) and Endophilin A3 (*D*) transfected cells. I_{rel} was calculated using the following equation $I_{rel} = (I\text{-}I_{min})/(I_{max}\text{-}I_{min})$ with I_{min} the current obtained without DC-EBIO and I_{max} the current obtained with 1000 µM DC-EBIO. The solid line through the data points was fitted by eye using a modified Hill equation, $I_{rel} = 1/(1 + (K_D / [\text{DC-EBIO}]))$. In control transfected cells (*C*) the dissociation constant K_D for DC-EBIO was 19 µM, for Endophilin A3 transfected cells (*D*) the K_D was 21 µM.

to calculate the Ca^{2+} concentration according to the equation mentioned in *Materials and Methods*. In order to determine the half-maximal activating Ca^{2+} concentration, the whole cell conductance was determined as the slope of the ramp current between -100 and -50 mV, was normalized to the largest slope of the ramp current observed during the experiment (g_{max}) and plotted against the simultaneously determined Ca^{2+} concentration for each experiment separately. Curves were fitted according to the equation $g/g_{max} = A/(1+(EC_{50}/[Ca^{2+}])^{nH})$, where g_{max} is the maximal conductance observed during the experiment, EC_{50} is the half-maximal activating Ca^{2+} concentration, and n_H is the Hill coefficient (Fig. 7, *bottom*). *A* was determined by the fit and was 1.0 for Fig. 7C and 1.3 for Fig. 7D. The results obtained showed a significant difference in the Ca^{2+} sensitivity of Endophilin A3 expressing cells (EC_{50} = 2.3 ± 0.04 µM, Hill coefficient = 5.6 ± 1.5) compared to that of control transfected cells (EC_{50} = 1 ± 0.1 µM, Hill coefficient = 4.6 ± 0.2). These results indicate that the interaction of Endophilin A3 with K_{Ca}2.3 channels makes the channels less sensitive to Ca^{2+}. Since the Ca^{2+}-sensitivity of K_{Ca}2.3 channels depends on the Ca^{2+}/Calmodulin binding site located at the C-terminus of the K_{Ca}2.3 channels [18-20] it might be feasible that other properties of K_{Ca}2.3 channels linked to that region of the channel like the activation by DC-EBIO [45] might also be modified.

Is the DC-EBIO sensitivity of K_{Ca}2.3 channels modified by the interaction with Endophilin A3?

Besides a change in Ca^{2+} sensitivity, which is conferred by the Ca^{2+}/Calmodulin binding site located at the C-terminus of K_{Ca}2.3 channels [18-20], one could also imagine a change in DC-EBIO sensitivity since this sensitivity has also been linked to the same region of the channel [45]. Therefore, we expressed Endophilin A3 in PC12 cells and investigated if there is an interference of Endophilin A3 with the DC-EBIO binding to K_{Ca}2.3 channels. To elicit K_{Ca}2.3 current, the pipette solution contained 1 µM free Ca^{2+}. DC-EBIO concentrations between 10 and 1000 µM were applied with the bath solution. Since the solution exchange takes about 15-30 s and could therefore mask the real time course of DC-EBIO action, we did not analyze the time course of DC-EBIO action but waited 150 s (10 voltage ramps, 15 s apart) in each solution before using the equilibrated current value for further analysis. Fig. 8 (*top left*) shows a representative ramp current trace through endogenous K_{Ca}2.3 channels of a control transfected PC12 cell in Cs^+ solutions containing different DC-EBIO concentrations. In Cs^+ solution without DC-EBIO the current amplitude was -308 pA at -80 mV and raised to –543 pA in Cs^+ solution with 30 µM DC-EBIO and to –646 pA in Cs^+ solution with 1000 µM DC-EBIO. In PC12 cells transfected with Endophilin A3 applying different DC-EBIO concentrations resulted also in an increase of the current amplitude (Fig. 8, *top right*). In Cs^+ solution without DC-EBIO the current amplitude at -80 mV was –25 pA and raised to-390 pA in Cs^+ solution with 30 µM DC-EBIO and to –610 pA in Cs^+ solution with 1000 µM DC-EBIO. It seems that, at saturating doses of DC-EBIO (1000 µM), the current amplitude of the control transfected cell (646 pA) was very similar to the current amplitude of the Endophilin A3 transfected cell (610 pA) indicating hardly any loss of functional channels in the membrane by Endophilin A3. In similar experiments we obtained in control transfected cells 770 ± 295 pA (n=18) and in Endophilin A3 transfected cells 640 ± 485 pA (n=25), therefore we cannot exclude a minor loss of functional channels of the membrane of Endophilin A3 transfected cells.

The DC-EBIO activation of K_{Ca}2.3 channels in control and in Endophilin A3 transfected cells can best be summarized in concentration-response curves shown in Fig. 8 (*bottom*). These concentration-response curves were generated from the current records shown in Fig. 8 (*top*) and similar records. The calculated relative currents (I_{rel} = $(I-I_{min})/(I_{max}-I_{min})$) were plotted against the DC-EBIO concentrations (Fig. 8, *bottom*). As there is no significant difference in the K_D values obtained in both concentration-response curves (19 µM for control, 21 µM for Endophilin A3 transfected PC12 cells) we conclude that the DC-EBIO sensitivity of K_{Ca}2.3 channels did not change through the interaction with Endophilin A3.

Discussion

In this study, we identified an interaction between Endophilin A3 and K_{Ca}2.3 channels using the yeast two-hybrid system, a pull-down assay, mouse brain slices and a mammalian cell line, PC12 cells. What is the impact of that interaction on the function of K_{Ca}2.3 channels? Like Endophilin A1 and A2, Endophilin A3 belongs to the family of endophilin proteins [29], which all play essential roles in endocytosis [46-51] and contain BAR and SH3 domains. The BAR domain of Endophilins induces positive curvature to the membrane. Therefore, Endophilins act prior to scission in clathrin-mediated endocytosis with differences in the functional specificity of the BAR domains [52]. Hence it could be that Endophilin A3 might remove K_{Ca}2.3 channels from the cell membrane. Patch-clamp experiments showed indeed that the number of functional channels decreased in PC12 cells transfected with Endophilin A3 compared to control cells. However, that decrease in number of functional channels was not a result of enhanced endocytosis since the elicited K_{Ca}2.3 current increased after application of DC-EBIO. Therefore, the action of Endophilin A3 on K_{Ca}2.3 channels was not a removal of channels from the cell membrane but a reduction of functional channels in the membrane by a change in the Ca^{2+} sensitivity of the K_{Ca}2.3 channels. It seems that binding of Endophilin A3 to the N-terminus of K_{Ca}2.3 channels can influence the binding/activity/coupling of CaM bound to the CaMBD (and adjacent channel segments from S6 to

the CaMBD) thereby influencing channel activity. This scenario is more intensively described in the following paragraph.

CaM/CaMBD and N/C interactions

K_{Ca} channels constitutively interact with calmodulin (CaM), which serves as Ca^{2+} sensor. A tetrameric K_{Ca}2.3 channel complex has at least 4 CaM bound. Bound CaM consists of an N- and a C-lobe with 2 Ca^{2+} binding EF hands on each lobe. CaM binds with its C-terminal domain (C-lobe) to around *h*K_{Ca}2.2 R419-L440, (*h*K_{Ca}2.3 R572-L593) and with its N-terminal domain (N-lobe) more distal around *h*K_{Ca}2.2 E469-Q487, (*h*K_{Ca}2.3 E622-Q640). The N- and C-lobe of CaM are connected by an α-helical CaM linker region (R74 to E83) containing T80 (according to NM_012518) the CK2 phosphorylation site. The binding of Ca^{2+} to the CaM N-lobe EF hands introduces the exposure of hydrophobic interfaces on the CaM N- and C-lobe that initiates the conformational changes that results in channel gating [53]. New protein crystal structures identified a region in K_{Ca}2.2 channels between S6 and the CaMBD important for the coupling in Ca^{2+} sensing by CaM and the mechanical opening of KCa channels [54]. In addition, this region is also involved in the binding of the DC-EBIO-related compound NS309 [54]. The authors could show that an intrinsically disordered fragment near S6, the so-called IDF region (*h*K_{Ca}2.2 A403-M412 equivalent to *h*K_{Ca}2.3 A556-M565) adapts a unique ordered conformation upon NS309 binding and that a C-terminal region of the K_{Ca}2.2 channel close to the membrane (E399-K402 equivalent to E552-K555 in *h*K_{Ca}2.3) interacts like a cuff with the CaM linker region after the proper CaM-CaMBD complex has been formed upon Ca^{2+} binding to CaM [54]. This interaction between the cuff and the CaM linker seems to contribute directly to the mechanical opening of K_{Ca}2.2 channels and can also explain that phosyphorylation of CaM by CK2 reduces Ca^{2+}-sensitivity of K_{Ca}2.2 channels by interfering with the mechanical coupling process rather than with Ca^{2+} binding to CaM [22, 23]. This scenario might also explain our results, where a change in Ca^{2+}-sensitivity of K_{Ca}2.3 by Endophilin A3 was observed, which could be overcome by DC-EBIO. In this case we would have to assume that Endophilin A3 either directly binds to the CaM linker region or changes the normal N-C interaction thereby not modulating the affinity of Ca^{2+} binding to CaM but changing the interaction of the cuff and the CaM linker. This modulation of the cuff/CaM linker interaction could be overcome by the binding of DC-EBIO to this region.

The N-terminal region of K_{Ca}2.3 channels, as shown here, does not only interact with Endophilin A3 but also with the C-terminus of K_{Ca}2.3 channels as had been described in yeast and in PC12 cells [30]. In those experiments, expressing an additional K_{Ca}2.3 N-terminal part in PC12 cells, the DC-EBIO sensitivity but not the Ca^{2+} sensitivity of the endogenously expressed K_{Ca}2.3 channels changed [30]. In that case the additional N-terminal fragment either directly or indirectly modulates the DC-EBIO binding site close to the CaM linker without modulating the affinity of Ca^{2+} binding to CaM or changing the interaction of the cuff and the CaM linker.

Taken together these and other observations confirm and extend earlier reports on the importance of N-C interactions, including those regarding the pH-sensitivity of Kir1.1 channels since amino acid changes of the interacting residues affected the pH-sensitivity of these channels [55].

Q19 vs Q14

The N-terminal region of the K_{Ca}2.3 channel contains two polyglutamine-repeats, linked by PXXP motives. The second polyglutamine repeat is a highly polymorphic. In the population repeat lengths from 12 to 28 were identified, the modal repeat length is coding for 19 Gln [25] or 18 Gln [56]. A K_{Ca}2.3$N_{(1\text{-}299),\ Q19}$ construct showed interaction of Endophilin A3 in our experiments whereas K_{Ca}2.3$N_{(1\text{-}274),\ Q14}$ did not. This difference could be due to the overall length of the tested N-tail (274 vs 299) or due to the difference in the polyglutamine stretch (Q14 vs Q19). An influence of the glutamine stretch would imply that individulas with shorter glutamine stretches would experience a higher K_{Ca}2.3 activity that might influence their cognitive performance especially in schizophrenia as has been suggested [56].

Physiological roles of the interaction of Endophilin A3 with K_{Ca}2.3 *channels*

The colocalization of K_{Ca}2.3 channels and Endophilin A3 in this paper is in agreement with previous studies determining either K_{Ca}2.3 alone or Endophilin A3 alone to synaptic membranes in neurons [57, 58]. Therefore, we can assume that the functional blockade of K_{Ca}2.3 channels by Endophilin A3, as shown in this study, may enhance signal transmission in neurons where K_{Ca}2.3 channels control synaptic plasticity as has been shown in mice, where the blockade of K_{Ca}2.3 channels with apamin, a specific SK channels blocker, facilitated memory encoding and learning [59, 60], and increased dopamine release [45, 61]. In addition, the activation of K_{Ca}2.3 channels with 1-EBIO or CyPPA impaired the encoding of object memory [62]. Therefore, our findings of Endophilin A3 reducing K_{Ca}2.3 channel function might be an important modulator of signal transduction in neurons. In addition, the interaction might have an influence on filopodia formation in neural stem cells [63], on podosome formation in microglia [64], in cancer cell migration [65-67], in trafficking of KCa2.3 channels via caveolae [68-71], and on lamellipodia protrusion [72].

Examples for Endophilin/Ion channel interactions from the literature

Another example about the importance of the interaction between endophilins and ion channels was the interaction of N-type voltage-gated Ca^{2+} channels with endophilins. Chen and colleagues [73] showed that Endophilin A2 interacted with the N-terminal part of voltage-gated Ca^{2+} channels and that this interaction was Ca^{2+}-dependent. The authors suggested an important role of that complex in coordinating the synaptic vesicle recycling by directly coupling to the endocytotic and exocytic machineries. Similar interactions have recently been shown between Endophilin A1 and A3 and N-type voltage-gated Ca^{2+} channels. Similar to the complex of Endophilin A2 with the Ca^{2+} channel, the formation of Endophilin A1 with the channel was also Ca^{2+} dependent. In contrast, Endophilin A3 interacted with N-type voltage-gated Ca^{2+} channels in a Ca^{2+}-independent manner [74]. It is, however, unclear whether this interaction between Endophilin A3 and the Ca^{2+} channel has any influence on channel activity.

Conclusion

The interaction of Endophilin A3 with the entire N-terminal part of K_{Ca}2.3 channels was shown in yeast as well as in pull-down assays. In addition, a colocalization of K_{Ca}2.3 channels and Endophilin A3 in mouse brain slices was demonstrated. The functional interaction of K_{Ca}2.3 channels and Endophilin A3 was also identified in PC12 cells which express endogenous K_{Ca}2.3 channels. Our results showed that Endophilin A3 reduced K_{Ca}2.3 currents but did not decrease the number of functional channels by endocytosis since K_{Ca}2.3 current increased again after DC-EBIO application. Endophilin A3 seems to reduce the Ca^{2+} sensitivity of K_{Ca}2.3 channels thereby decreasing the number of conducting channels in the membrane under physiological conditions. These results indicate that the interaction of Endophilin A3 with K_{Ca}2.3 channels also occurs in mammalian cells and might have an important impact on the function of cells where Endophilin A3 and K_{Ca}2.3 channels are colocalized.

Abbreviations

SK channels, small conductance Ca^{2+} activated K^+ channel, K_{Ca}2.1-K_{Ca}2.3 (SK1-3 encoded by KCNN1-KCNN3), member of the SK channel family; fAHP (fast afterhyperpolarisation); AP (action potential); CaMBD (calmodulin binding domain); CaM (calmodulin); DC-EBIO (5,6-Dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one); PCR (polymerase chain reaction); DMEM (Dulbecco's modified Eagle's medium); FCS (fetal calf serum); HS (horse serum); GFP (green fluorescent protein); BSA (bovine serum albumin); polyglutamine (polyQ); proline-rich region (PRR); Huntingtin protein (Htt); N-C interaction (interaction

between the cytoplasmic N-terminal with the cytoplasmic C-terminal part of the channel); Alternative names for Endophilin A3 (Gene name: SH3GL3 or CNSA3 or SH3D2C); Endophilin 3, EEN-B2, Endophilin-3, SH3 domain protein 2C, SH3 domain-containing GRB2-like protein 3.

Acknowledgements

We thank Ms Katharina Ruff and Ms Sabine Conrad for excellent technical assistance. This work was supported by grants from the DFG (Gr848 16-1 and 17-1 to SG).

References

1 Blatz AL, Magleby KL: Single apamin-blocked Ca-activated K^+ channels of small conductance in cultured rat skeletal muscle. Nature 1986;323:718-720.
2 Barfod ET, Moore AL, Lidofsky SD: Cloning and functional expression of a liver isoform of the small conductance Ca^{2+}-activated K^+ channel SK3. Am J Physiol Cell Physiol 2001;280:C836-C842.
3 Köhler M, Hirschberg B, Bond CT, Kinzie JM, Marrion NV, Maylie J, Adelman JP: Small-Conductance, Calcium-Activated Potassium Channels from Mammalian Brain. Science 1996;273:1709-1714.
4 Lingle CJ, Solaro CR, Prakriya M, Ding JP: Calcium-activated potassium channels in adrenal chromaffin cells. Ion Channels 1996;4:261-301.
5 Lomax RB, Warhurst G, Sandle GI: Characteristics of two basolateral potassium channel populations in human colonic crypts. Gut 1996;38:243-247.
6 Neher E: Help from fast synapses. Nature 1996;383:393-394.
7 Ro S, Hatton WJ, Koh SD, Horowitz B: Molecular properties of small-conductance Ca^{2+}-activated K^+ channels expressed in murine colonic smooth muscle. Am J Physiol Gastrointest Liver Physiol 2001;281:G964-973.
8 Sailer CA, Hu H, Kaufmann WA, Trieb M, Schwarzer C, Storm JF, Knaus HG: Regional differences in distribution and functional expression of small-conductance Ca^{2+}-activated K^+ channels in rat brain. J Neurosci 2002;22:9698-9707.
9 Sailer CA, Kaufmann WA, Marksteiner J, Knaus HG: Comparative immunohistochemical distribution of three small-conductance Ca^{2+}-activated potassium channel subunits, SK1, SK2, and SK3 in mouse brain. Mol Cell Neurosci 2004;26:458-469.
10 Shmukler BE, Bond CT, Wilhelm S, Bruening-Wright A, Maylie J, Adelman JP, Alper SL: Structure and complex transcription pattern of the mouse SK1 K(Ca) channel gene, KCNN1. Biochim Biophys Acta 2001;1518:36-46.
11 Stocker M: Ca^{2+}-activated K^+ channels: molecular determinants and function of the SK family. Nat Rev Neurosci 2004;5:758-770.
12 Stocker M, Pedarzani P: Differential distribution of three Ca^{2+}-activated K^+ channel subunits, SK1, SK2, and SK3, in the adult rat central nervous system. Mol Cell Neurosci 2000;15:476-493.
13 Tamarina NA, Wang Y, Mariotto L, Kuznetsov A, Bond C, Adelman J, Philipson LH: Small-conductance calcium-activated K^+ channels are expressed in pancreatic islets and regulate glucose responses. Diabetes 2003;52:2000-2006.
14 Tse A, Hille B: GnRH-induced Ca^{2+} oscillations and rhythmic hyperpolarizations of pituitary gonadotropes. Science 1992;255:462-464.
15 Tse A, Tse FW, Hille B: Modulation of Ca^{2+} oscillation and apamin-sensitive, Ca^{2+}-activated K^+ current in rat gonadotropes. Pflugers Arch 1995;430:645-652.
16 Sah P: Ca^{2+}-activated K^+ currents in neurons: types, physiological roles and modulation. Trends Neurosci 1996;19:150-154.
17 Maciaszek JL, Soh H, Walikonis RS, Tzingounis AV, Lykotrafitis G: Topography of Native SK Channels Revealed by Force Nanoscopy in Living Neurons. J Neurosci 2012;32:11435–11440.
18 Adelman JP, Maylie J, Sah P: Small-conductance Ca^{2+}-activated K_channels: form and function. Annu Rev Physiol 2012;74:245–269.

19 Schumacher M, Rivard A, Bachinger H, Adelman JP: Structure of the gating domain of a Ca^{2+}-activated K^+ channel complexed with Ca^{2+}/calmodulin. Nature 2001;410:1120–1124.
20 Maylie J, Bond CT, Herson PS, Lee WS, Adelman JP: Small conductance Ca^{2+}-activated K^+ channels and calmodulin. J Physiol 2004;554:255-261.
21 Halling DB, Kenrick SA, Riggs AF, Aldrich RW: Calcium-dependent stoichiometries of the KCa2.2 (SK) intracellular domain/calmodulin complex in solution. J Gen Physiol 2014;143:231-252.
22 Bildl W, Strassmaier T, Thurm H, Andersen J, Eble S, Oliver D, Knipper M, Mann M, Schulte U, Adelman JP, Fakler B: Protein kinase CK2 is coassembled with small conductance Ca^{2+}-activated K^+ channels and regulates channel gating. Neuron 2004;43:847-858.
23 Allen D, Fakler B, Maylie J, Adelmann JP: Organization and Regulation of Small Conductance Ca^{2+}-activated K^+ Channel Multiprotein Complexes. J Neurosci 2007;27:2369-2376.
24 Pierce SL, Kresowik JDK, Lamping KG, England SK: Overexpression of SK3 Channels Dampens Uterine Contractility to Prevent Preterm Labor in Mice. Biol Reprod 2008;78:1058–1063.
25 Chandy KG, Fantino E, Wittekindt O, Kalman K, Tong LL, Ho TH, Gutman GA, Crocq MA, Ganguli R, Nimgaonkar V, Morris-Rosendahl DJ, Gargus JJ: Isolation of a novel potassium channel gene *h*SKCa3 containing a polymorphic CAG repeat: a candidate for schizophrenia and bipolar disorder? Mol Psychiatry 1998;3:32-37.
26 Sittler A, Wälter S, Wedemeyer N, Hasenbank R, Scherzinger E, Eickhoff H, Bates GP, Lehrach H, Wanker EE: SH3GL3 Associates with the Huntingtin Exon 1 Protein and Promotes the Formation of Polygln-Containing Protein Aggregates. Mol Cell 1998;2:427–436.
27 Gao YG, Yan XZ, Song AX, Chang YG, Gao XC, Jiang N, Zhang Q, Hu HY: Structural Insights into the Specific Binding of Huntingtin Proline-rich Region with the SH3 and WW Domains. Structure 2006;14:1755-1765.
28 Li SH and Li XJ: Huntingtin-protein interactions and the pathogenesis of Huntington's disease. Trends Genetic 2004;20:146-154.
29 Giachino C, Lantelme E, Lanzetti L, Saccone S, Della Valle G, Migone N: A Novel SH3-Containing Human Gene Family Preferentially Expressed in the Central Nervous System. Genomics 1997;41:427–434.
30 Frei E, Spindler I, Grissmer S, Jäger H: Interaction of N-Terminal and C-Terminal Parts of the Small Conductance Ca^{2+} Activated K^+ Channel, *h*SK3. Cell Physiol Biochem 2006;18:165-176.
31 Scherzinger E, Lurz R, Turmaine M, Mangiarini L, Hollenbach B, Hasenbank R, Bates GP, Davies SW, Lehrach H, Wanker EE: Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. Cell 1997;90:549-458.
32 Wanker EE, Rovira C, Scherzinger E, Hasenbank R, Wälter S, Tait D, Colicelli J, Lehrach H: HIP-I: a huntingtin interacting protein isolated by the yeast two-hybrid system. Hum Mol Genet 1997;6:487-495.
33 Vojtek AB, Hollenberg SM, Cooper JA: Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell 1993;74:205-214.
34 Wachter B, Schürger S, Rolinger J, von Ameln-Mayerhofer A, Berg D, Wagner HJ, Küppers E: Effect of 6-hydroxydopamine (6-OHDA) on proliferation of glial cells in the rat cortex and striatum: evidence for de-differentiation of resident astrocytes. Cell Tissue Res 2010;342:147-160.
35 Greene LA, Tischler AS: PC12 Pheochromocytoma cultures in neurobiological research. in: S. Fedoroff and L. (eds): Hertz, Advances in Cellular Neurobiology. Academic Press, New York, 1982, vol. 3, pp 374-414.
36 Sambrook J, Fritsch EF, Maniatis: Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.
37 Wanker EE, Rovira C, Scherzinger E, Hasenbank R, Wälter S, Tait D, Colicelli J, Lehrach H: HIP-1: a huntingtin interacting protein isolated by the yeast two-hybrid system. Hum Mol Genet 1997;6:487–495.
38 Li B, Fields S: Identification of mutations in p53 that affect its binding to SV40 large T antigen by using the yeast two-hybrid system. FASEB J 1993;7:957-963.
39 Probst MR, Fan C, Tessier-Lavigne M, Hankinson O: Two Murine Homologs of the *Drosophila* Single-minded Protein That Interact with the Mouse Aryl Hydrocarbon Receptor Nuclear Translocator Protein. J Biol Chem 1997;272:4451-4457.
40 Kido M, Yamanaka K, Mitani T, Niki H, Ogura T, Hiraga S: RNase E Polypeptides Lacking a Carboxyl-terminal Half Suppress a *mukB* mutation in *Escherichia coli*. J. Bacteriol 1996;178:3917-3925.
41 Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch 1981;391:85-100.

42 Rauer H, Grissmer S: Evidence for an internal phenylalkylamine action on the voltage-gated potassium channel Kv1.3. Mol Pharmacol 1996;50:1625-1634.
43 McCloskey MA, Cahalan MD: G protein control of potassium channel activity in a mast cell line. J Gen Physiol 1990;95, 205-227.
44 Wittekindt OH, Visan V, Tomita H, Imtiaz F, Gargus JJ, Lehmann-Horn F, Grissmer S, Morris-Rosendahl DJ: An Apamin- and Scyllatoxin-Insensitive Isoform of the Human SK3 Channel. Mol Pharmacol 2004;65:788-801.
45 Pedarzani P, Mosbacher J, Rivard A, Cingolani LA, Oliver D, Stocker M, Adelman JP, Fakler B: Control of electrical activity in central neurons by modulating the gating of small conductance Ca^{2+}-activated K^+ channels. J Biol Chem 2001;276:9762-9769.
46 Guichet A, Wucherpfennig T, Dudu V, Etter S, Wilsch-Brauniger M, Hellwig A, González-Gaitán M, Huttner WB, Schmidt AA: Essential role of endophilin A in synaptic vesicle budding at the Drosophilaneuromuscular junction. EMBO Journal 2002;21:1661–1672.
47 Verstreken P, Kjaerulff O, Lloyd TE, Atkinson R, Zhou Y, Meinertzhagen IA, Bellen HJ: Endophilin mutations block clathrin-mediated endocytosis but not neurotransmitter release. Cell 2002;109:101–112.
48 Ringstad N, Nemoto Y, De Camilli P: The SH3p4/Sh3p8/SH3p13 protein family: Binding partners for synaptojanin and dynamin via a Grb2-like Src homology 3 domain. Proceedings of the National Academy of Sciences USA 1997;94:8569–8574.
49 De Gois S, Jeanclos E, Morris M, Grewal S, Varoqui H, Erickson JD: Identification of endophilins 1 and 3 as selective binding partners for VGLUT1 and their co-localizationin neocortical glutamatergic synapses: Implications for vesicularglutamate transporter trafficking and excitatory vesicle formation. Cell Mol Neurobiol 2006;26:679–693.
50 Fabian-Fine R, Verstreken P, Hiesinger PR, Horne JA, Kostyleva R, Zhou Y, Bellen HJ, Meinertzhagen IA: Endophilin promotes a late step in endocytosis at glial invaginations in Drosophilaphotoreceptor terminals. J Neurosci 2003;23:10732-10744.
51 Schuske KR, Richmond JE, Matthies DS, Davis WS, Runz S, Rube DA, van der Bliek AM, Jorgensen EM: Endophilin is required for synaptic vesicle endocytosis by localizing synaptojanin. Neuron 2003;40:749–762.
52 Bai J, Hu Z, Dittman JS, Pym EC, Kaplan JM: Endophilin functions as a membrane-bending molecule and is delivered to endocytic zones by exocytosis. Cell 2010;143:430-441.
53 Zhang M, Pascal JM, Schumann M, Armen RS, Zhang J-F: Identification of the functional binding pocket for compounds targeting small-conductance Ca^{2+}-activated potassium channels. Nat Commun doi: 10.1038/ncomms2017.
54 Zhang M, Pascal JM, Zhang J-F: Unstructured to structured transition of an intrinsically disordered protein peptide in coupling Ca^{2+}-sensing and SK cahnnel activation. PNAS 2013;110:4828-4833.
55 Rapedius M, Haider S, Browne KF, Shang L, Sansom MS, Baukrowitz T, Tucker SJ: Structural and functional analysis of the putative pH sensor in the Kir1.1 (ROMK) potassium channel. EMBO Rep 2006;7:611-616.
56 Grube S, Gerchen MF, Adamico B, Pardo LA, Martin S, Malzahn D, Papiol S, Begemann M, Ribbe K, Fridrichs H, Radyushkin KA, Müller M, Benseler F, Riggert J, Falkai P, Bickeböller H, Nave K-A, Brose N, Stühmer W, Ehrenreich H: A CAG repeat polymorphism of KCNN3 predicts SK3 channel function and cognitive performance in schizophrenia. EMBO Mol Med 2011;3:309-319.
57 Obermair GJ, Kaufmann WA, Knaus HG, Flucher BE: The small conductance Ca^{2+}-activated K^+ channel SK3 is localized in nerve terminals of excitatory synapses of cultured mouse hippocampal neurons. Eur J Neurosci 2003;17:721-731.
58 Roncarati R, Di Chio M, Sava A, Terstappen GC, Fumagalli G: Presynaptic localization of the small conductance calcium-activated potassiumchannel SK3 at the neuromuscular junction. Neuroscience 2001;104:253–262.
59 Messier C, Mourre C, Bontempi B, Sif J, Lazdunski M, Destrade C: Effect of apamin, a toxin that inhibits Ca^{2+}-dependent K^+ channels, on learning and memory processes. Brain Res 1991;551:322–326.
60 Deschaux O, Bizot JC, Goyffon M: Apamin improves learning in an object recognition task in rats. Neurosci Lett 1997;222:159–162.
61 Stocker M, Krause M, Perdarzani P: An apamin-sensitive Ca^{2+}-activated K^+ current in hippocampal pyramidal neurons. Proc Natl Acad Sci U S A, 1999;96:4662-4667.

62 Vick KA 4th, Guidi M, Stackman RW Jr: In vivo pharmacological manipulation of small conductance Ca^{2+}-activated K^+ channels influences motor behavior, object memory and fear conditioning. Neuropharm 2010;58:650-659.
63 Liebau S, Steinestel J, Linta L, Kleger A, Storch A, Schoen M, Steinestel K, Proepper C, Bockmann J, Schmeisser MJ, Boeckers TM: An SK3 channel/nWASP/Abi-1 complex is involved in early neurogenesis. PLoSOne 2011;6:e18148. doi: 10.1371/journal.pone.0018148.
64 Siddiqui TA, Lively S, Vincent C, Schlichter LC: Regulation of podosome formation, microglial migration and invasion by Ca^{2+}-signaling molecules expressed in podosomes. J Neuroinflam 2012;9:250.doi: 10.1186/1742-2094-9-250.
65 Chantôme A, Potier-Cartereau M, Clarysse L, Fromont G, Marionneau-Lambot S, Guéguinou M, Pagès JC, Collin C, Oullier T, Girault A, Arbion F, Haelters JP, Jaffrès PA, Pinault M, Besson P, Joulin V, Bougnoux P, Vandier C: Pivotal role of the lipid Raft SK3-Orai1 complex in human cancer cell migration and bone metastases. Cancer Res 2013;73:4852-4861.
66 Girault A, Haelters JP, Potier-Cartereau M, Chantôme A, Jaffrés PA, Bougnoux P, Joulin V, Vandier C: Targeting SKCa channels in cancer: potential new therapeutic approaches. Curr Med Chem 2012;19:697-713.
67 Potier M, Joulin V, Roger S, Besson P, Jourdan ML, Leguennec JY, Bougnoux P, Vandier C: Identification of SK3 channel as a new mediator of breast cancer cell migration. Mol Cancer Ther 2006;5:2946-2953.
68 Pani B, Singh BB: Lipidrafts/caveolae as microdomains of calcium signaling. Cell Calcium 2009;45:625-633.
69 Absi M, Burnham MP, Weston AH, Harno E, Rogers M, Edwards G: Effects of methyl beta-cyclodextrin on EDHF responses in pig and rat arteries; association between SK(Ca) channels and caveolin-rich domains. Br J Pharmacol 2007;151:332-340.
70 Balut CM, Hamilton KL, Devor DC: Trafficking of Intermediate (KCa3.1) and Small (KCa2.x) Conductance, Ca^{2+}-Activated K^+ Channels: a Novel Target for Medicinal Chemistry Efforts? Chem Med Chem 2012;7:1741-1755.
71 Gao Y, Bertuccio CA, Balut CM, Watkins SC, Devor DC: Dynamin- and Rab5-Dependent Endocytosis of a Ca^{2+}-Activated K^+ Channel. KCa2.3. PLoS ONE 2012;7:e44150. doi:10.1371/journal.pone.0044150.
72 Vehlow A, Soong D, Vizcay-Barrena G, Bodo C, Law AL, Perera U, Krause M: Endophilin, Lamellipodin, and Mena cooperate to regulate F-actin-dependent EGF-receptor endocytosis. EMBO J 2013;32:2722-2734.
73 Chen Y, Deng L, Maeno-Hikichi Y, Lai M, Chang S, Chen G, Zhang JF: Formation of an endophilin-Ca^{2+} channel complex is critical for clathrin-mediated synaptic vesicle endocytosis. Cell 2003;115:37-48.
74 Tian Q, Zhang J-F, Fan J, Song Z, Chen Y: Endophilin isoforms have distinct characteristics in interactions with N-type Ca^{2+} channels and dynamin I. Neurosci Bull 2012;28:483–492.