

Original Paper

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

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## 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  $\mu$ M free  $Ca^{2+}$ . **Results:** Whole-cell measurements of PC12 cells transfected with Endophilin A3 showed a reduction of  $K_{Ca}2.3$  specific  $Ca^{2+}$  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  $\mu$ M), 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.

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## 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<sub>(1-274), Q14</sub> ( $K_{Ca}2.3$  N-terminal region with 14Q), and the pcDNA3SK3N<sub>(1-299), Q19</sub> ( $K_{Ca}2.3$  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<sub>(1-299), Q19</sub>; pDNA-BD-SK3N<sub>(1-274), Q14</sub>, EcoRI XhoI fragments from pLexA SK3N<sub>(1-299), Q19</sub> and pLexA SK3N<sub>(1-274), Q14</sub> [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<sub>(1-299)</sub> 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 Lipofectamine™ 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 [ $MAT\alpha$ ,  $his3$ ,  $trp1$ ,  $ura3$ ,  $lexA_{op(x8)}$ ,  $lys2$ ,  $lexA_{op(x4)}$  -  $HIS3$ ] [37] was transformed with the reporter plasmid p8oplacZ ( $lexA_{op(x8)}$   $ura3Amp^r$ ) (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.

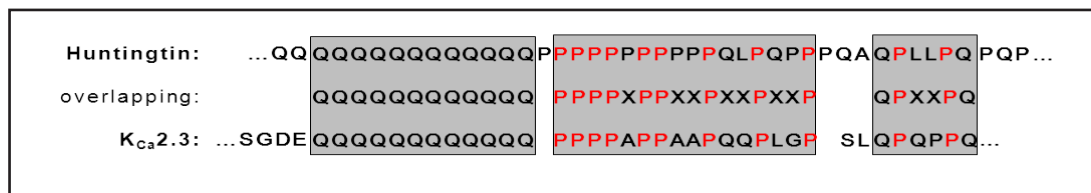
#### Patch-clamp experiments

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  $\mu M$  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  $\mu M$  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  $\mu l$  of tip solution (containing 50  $\mu M$  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  $\mu M$  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  $\mu M$  free  $Ca^{2+}$ , pH 7.2). All values presented here are mean  $\pm$  SD with n the number of independent observations.

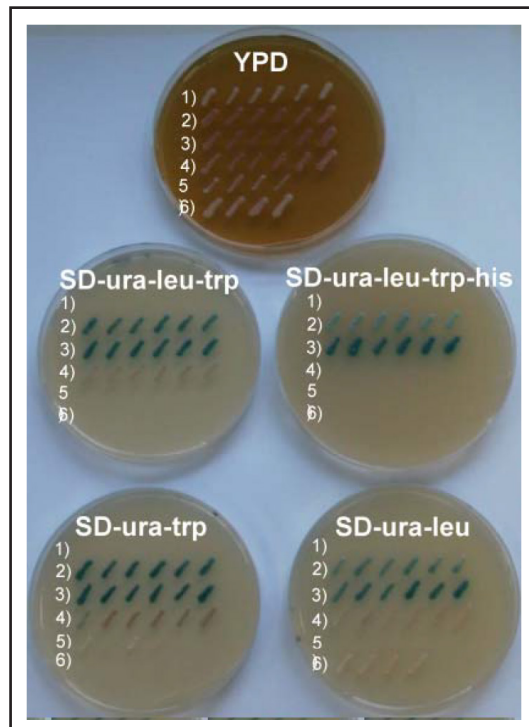
#### Measuring intracellular $Ca^{2+}$

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  $[Ca^{2+}]_i = K_{eff} \times (R - R_{min}) / (R_{max} - R)$ , 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  $\beta$ -galactosidase. Row 1: untransformed yeast strain L40; Row 2: DNA-BD-SIM; AD-ARNT (positive control); Row 3: DNA-BD-  $K_{Ca}2.3N_{(1-299),Q19}$ ; AD-Endophilin A3; Row 4: DNA-BD-  $K_{Ca}2.3N_{(1-274),Q14}$ ; AD-Endophilin A3; Row 5: DNA-BD-  $K_{Ca}2.3N_{(1-299),Q19}$ ; 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  $\mu M$ ), respectively, and  $K_{eff}$  is the effective dissociation constant. For calibration, cells were perfused with internal solution containing minimal, saturating, and 0.5  $\mu M$   $[Ca^{2+}]$  and 50  $\mu M$  fura-2 (Molecular Probes, Eugene, OR). Values for  $K_{eff}$ ,  $R_{min}$ , and  $R_{max}$  were calculated to be 1.134  $\mu M$ , 0.1, and 1.74.

## Results

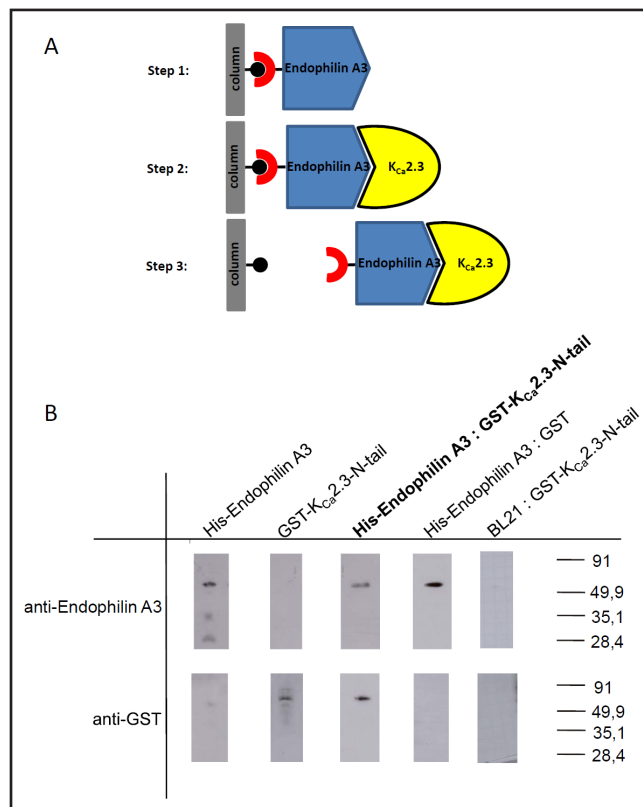
The N-terminal part of the  $K_{Ca}2.3$  channel is unique within the  $K_{Ca}$  channel family. It is about two times longer compared to  $K_{Ca}2.1$  and  $K_{Ca}2.2$  channels and has a high similarity to Huntingtin (Fig. 1). Since Huntingtin was shown to interact with Endophilin A3 [26] we wanted 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  $K_{Ca}2.3N_{(1-299),Q19}$  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: Elution 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, elution 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.



$K_{Ca}2.3N_{(1-299),Q19}$  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  $K_{Ca}2.3N_{(1-274),Q14}$  did not interact with Endophilin A3 (row 4).

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

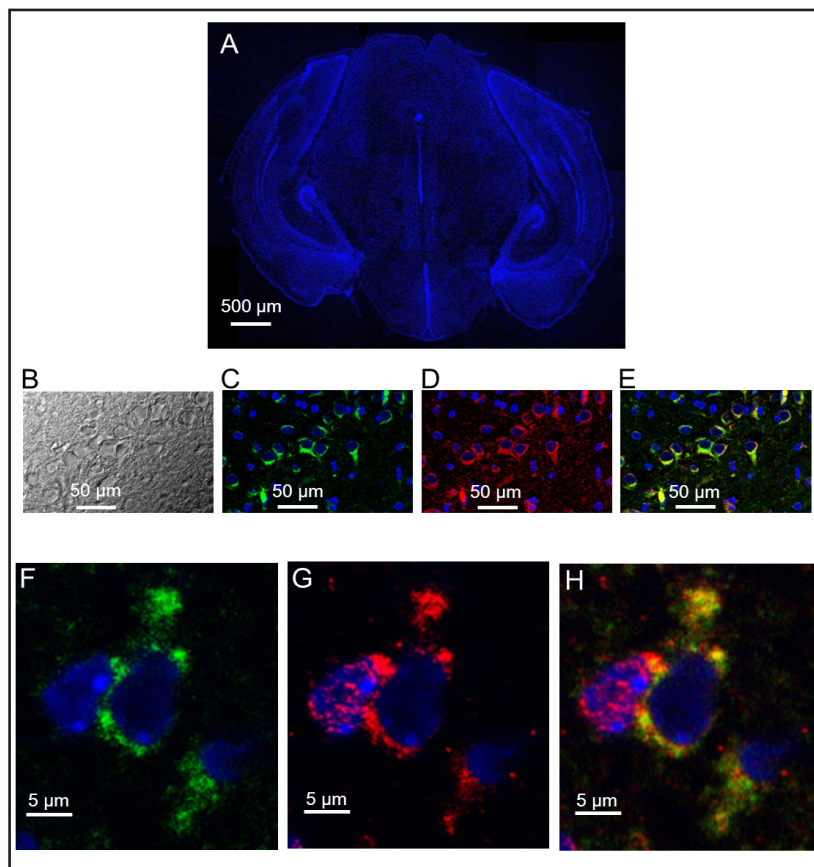
#### Pull-down experiments

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  $K_{Ca}2.3$

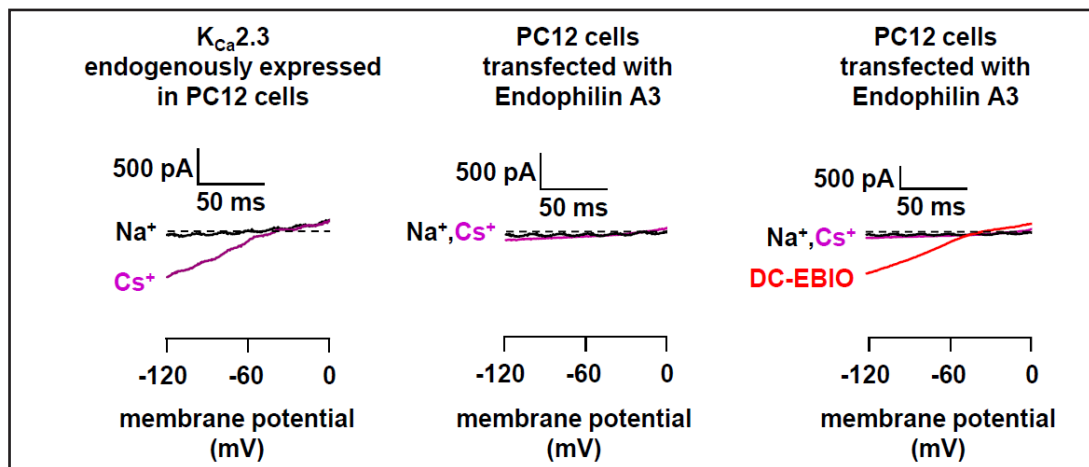
**Fig. 4.** Immunohistochemical costaining of  $K_{Ca}2.3$  channels with Endophilin A3 in mouse brain slices. (A) Overview of a coronal slice through a postnatal (P3) mouse brain corresponding Bregma -3.6 mm; nuclei are depicted in blue (DAPI). (B-E) Higher magnification of the dentate gyrus in A using 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.

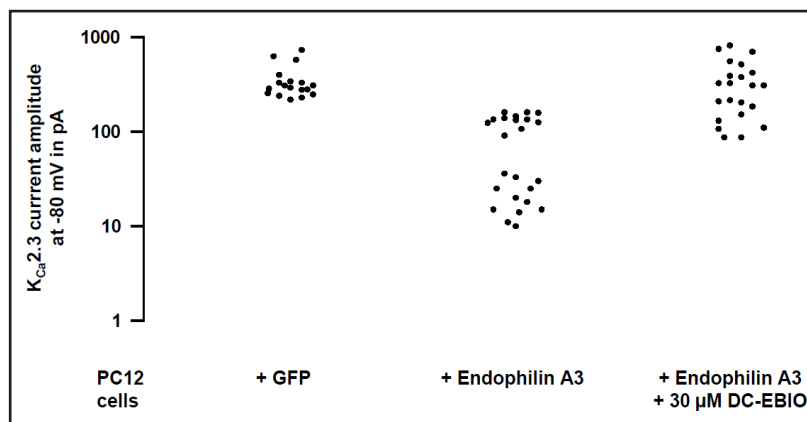
*Does Endophilin A3 functionally interact with  $K_{Ca}2.3$  in mammalian cells?*

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  $\mu M$  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  $\mu M$  DC-EBIO (*right*).  $K_{Ca}2.3$  channels were activated by pipette solutions containing 1  $\mu M$  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  $\mu M$  DC-EBIO (*right*).

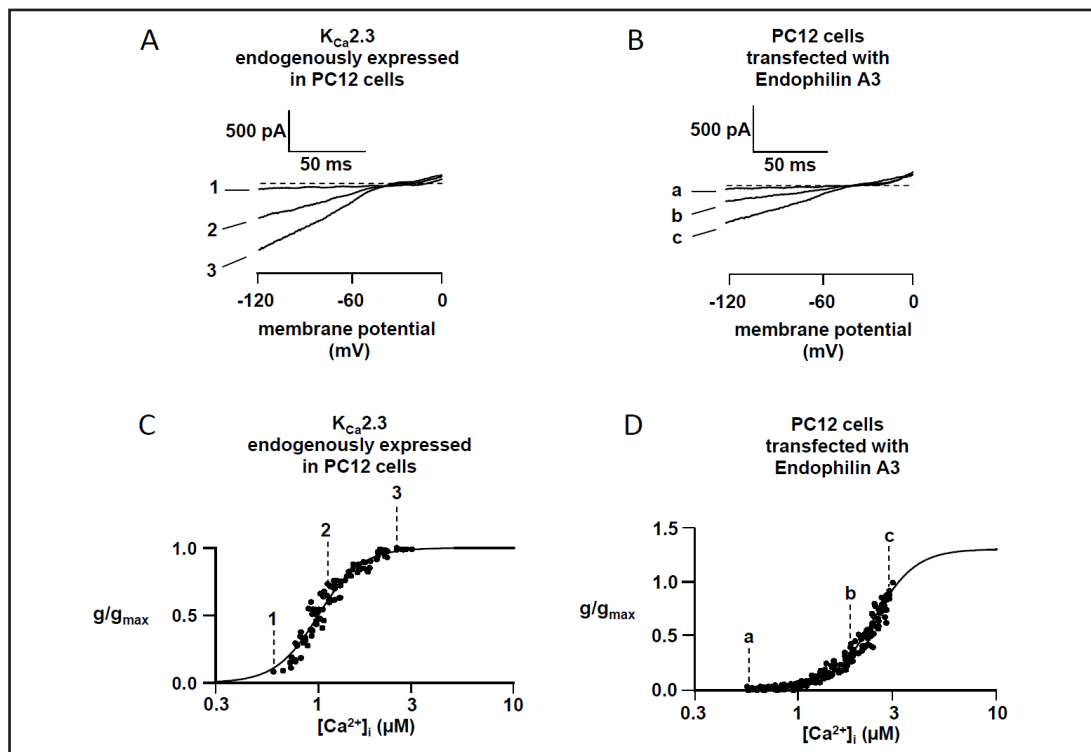


a value close to that obtained in control transfected cells with  $Cs^+$  solution alone (compare Fig. 5, *left* with *right*).

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  $-350 \pm 150$  pA (mean  $\pm$  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  $-80 \pm 60$  pA ( $n=24$ ).

The current amplitudes of PC12 cells transfected with Endophilin A3 in the presence of 30  $\mu M$  DC-EBIO were comparable to those obtained in control transfected PC12 cells in  $Cs^+$ -solution without DC-EBIO ( $-360 \pm 270$  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



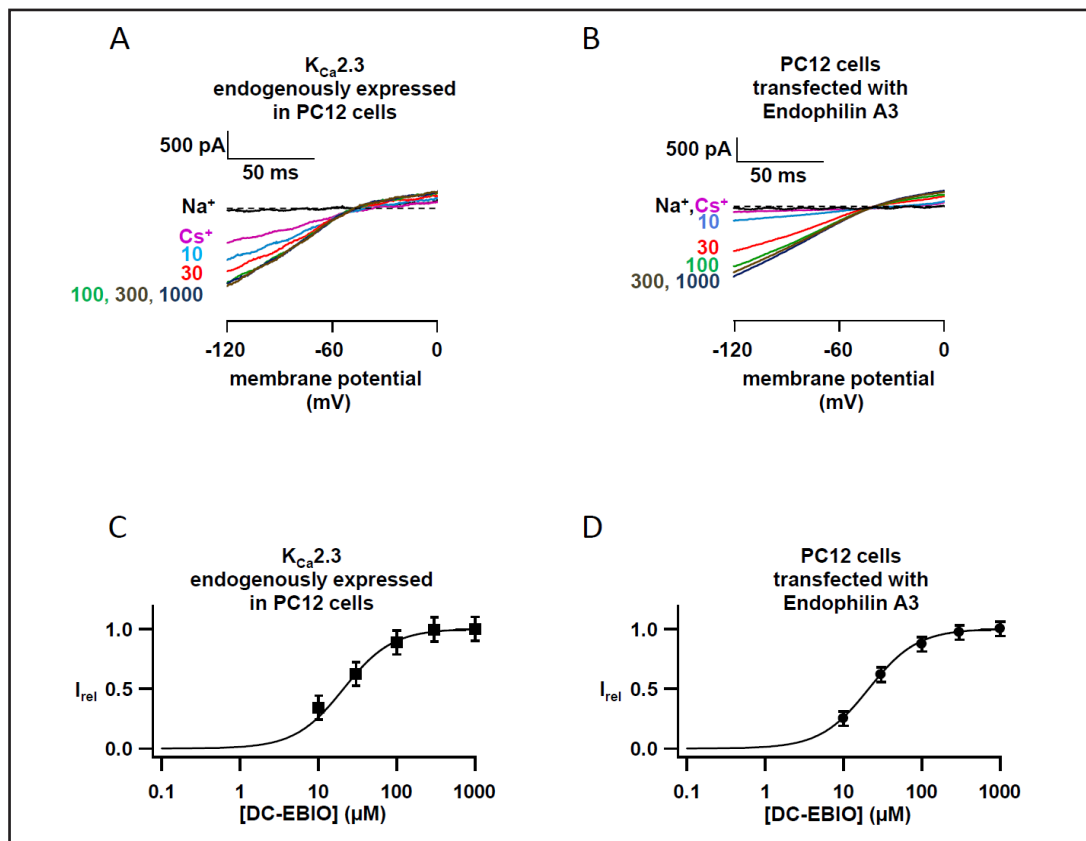


**Fig. 7.** Activation of  $K_{Ca}2.3$  channels in PC12 cells control transfected (GFP) or transfected with Endophilin A3 by internal  $Ca^{2+}$ . Membrane potentials were ramped from -120 to +60 mV in 200 ms. In parallel, the internal  $Ca^{2+}$  concentration was measured by the fura-2 method.  $Ca^{2+}$  aspartate solution was used as external solution. Pipettes were filled with 2 to 4  $\mu l$  of tip solution containing 10 mM EGTA without any  $Ca^{2+}$ , which was overlaid with pipette-solution containing 3  $\mu M$  free  $Ca^{2+}$  and 1 mM EGTA (internal solutions contained 50  $\mu M$  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  $g/g_{max} = A/(1 + (EC_{50}/[Ca^{2+}])^{n_H})$ , 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  $1.0 \pm 0.1 \mu M$  and the Hill coefficient  $4.6 \pm 0.2$ . For Endophilin A3 transfected cells the  $EC_{50}$  was  $2.3 \pm 0.04 \mu M$  and the Hill coefficient  $5.6 \pm 1.5$ .

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  $K_{Ca2.3}$  currents with different DC-EBIO concentrations. (A) representative endogenous  $K_{Ca2.3}$  currents in control (GFP) transfected cells. (B) representative endogenous  $K_{Ca2.3}$  currents in cells transfected with Endophilin A3. (C,D) Concentration-response curves for the activation of  $K_{Ca2.3}$  current by different DC-EBIO concentrations (in comparison to the activation of  $K_{Ca2.3}$  current by 1000  $\mu M$  DC-EBIO) of control-GFP (C) and Endophilin A3 (D) transfected cells.  $I_{rel}$  was calculated using the following equation  $I_{rel} = (I - I_{min}) / (I_{max} - I_{min})$  with  $I_{min}$  the current obtained without DC-EBIO and  $I_{max}$  the current obtained with 1000  $\mu M$  DC-EBIO. The solid line through the data points was fitted by eye using a modified Hill equation,  $I_{rel} = 1 / (1 + (K_D / [DC-EBIO])^n)$ . In control transfected cells (C) the dissociation constant  $K_D$  for DC-EBIO was 19  $\mu M$ , for Endophilin A3 transfected cells (D) the  $K_D$  was 21  $\mu M$ .

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  $g/g_{max} = A / (1 + (EC_{50} / [Ca^{2+}])^{n_H})$ , 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 ( $EC_{50} = 2.3 \pm 0.04 \mu M$ , Hill coefficient =  $5.6 \pm 1.5$ ) compared to that of control transfected cells ( $EC_{50} = 1 \pm 0.1 \mu M$ , Hill coefficient =  $4.6 \pm 0.2$ ). These results indicate that the interaction of Endophilin A3 with  $K_{Ca2.3}$  channels makes the channels less sensitive to  $Ca^{2+}$ . Since the  $Ca^{2+}$ -sensitivity of  $K_{Ca2.3}$  channels depends on the  $Ca^{2+}$ /Calmodulin binding site located at the C-terminus of the  $K_{Ca2.3}$  channels [18-20] it might be feasible that other properties of  $K_{Ca2.3}$  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  $\mu M$  free  $Ca^{2+}$ . DC-EBIO concentrations between 10 and 1000  $\mu M$  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  $\mu M$  DC-EBIO and to -646 pA in  $Cs^+$  solution with 1000  $\mu M$  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  $\mu M$  DC-EBIO and to -610 pA in  $Cs^+$  solution with 1000  $\mu M$  DC-EBIO. It seems that, at saturating doses of DC-EBIO (1000  $\mu M$ ), 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  $770 \pm 295$  pA ( $n=18$ ) and in Endophilin A3 transfected cells  $640 \pm 485$  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  $\mu M$  for control, 21  $\mu M$  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  $hK_{Ca}2.2$  R419-L440, ( $hK_{Ca}2.3$  R572-L593) and with its N-terminal domain (N-lobe) more distal around  $hK_{Ca}2.2$  E469-Q487, ( $hK_{Ca}2.3$  E622-Q640). The N- and C-lobe of CaM are connected by an  $\alpha$ -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  $K_{Ca}$  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 ( $hK_{Ca}2.2$  A403-M412 equivalent to  $hK_{Ca}2.3$  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  $hK_{Ca}2.3$ ) 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 phosphorylation 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 Glns [25] or 18 Glns [56]. A  $K_{Ca}2.3N_{(1-299), Q19}$  construct showed interaction of Endophilin A3 in our experiments whereas  $K_{Ca}2.3N_{(1-274), Q14}$  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 individual 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-2H-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.

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