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Interactions of N-Terminal and C-Terminal Parts of the Small Conductance Ca^{2+} Activated K^+ Channel, *h*SK3

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Key Words

SK Channels • KCNN1 • KCNN2 • KCNN3 • N-C interactions • N-N interaction • 1-EBIO • Yeast two hybrid experiments • Electrophysiology • Ca^{2+} Measurements

Abstract

Ca^{2+} activated K^+ channels modulate the afterhyperpolarization in neurons. Using a variety of different techniques we obtained information about the function of N- and C-terminal parts of the Ca^{2+}-activated K^+ channel, SK3. By means of the yeast two hybrid technique we found an interaction between N-C and N-N- terminal parts of SK3. The strong N-C and N-N interaction was specific for SK3 and could not be observed for SK1 and SK2. Possibly a homotetrameric assembly of SK3 is favored in tissues were all SK channels are expressed. In addition, the interaction in SK3 was independent of the length of the polymorphic glutamine repeat in the N-terminus of SK3. Electrophysiological investigations showed that expression of amino acids 1-299 of SK3 (SK3N_299) modified the 1-EBIO pharmacology of endogenous SK3 channels in PC12 cells without affecting the Ca^{2+}-sensitvity. The activation by 0.5 mM 1-EBIO in cells expressing amino acids 1-299 of SK3 was reduced by 32% in comparison to control experiments. Considering the N-C interaction in yeast, we conclude that the sensitivity of SK3 channels to 1-EBIO was modified by N-C interactions with SK3N_299. Therefore we conclude that N-C interactions influence SK3 channel function.

Introduction

Small conductance Ca^{2+} activated K^+ channels (SK channels) are widely expressed in the brain [1-3] where they contribute to the afterhyperpolarization (AHP) following an action potential (AP) in neurons. Functional SK channels are tetramers of 4 α- subunits and calmodulin (CaM) constitutively bound to the CaM binding domain (CaMBD) in the cytoplasmic C-terminal part of each α-subunit [4, 5]. The α-subunits of SK1-SK3 are highly homologous within their transmembrane domains, but diverge in their N-terminal parts. Compared to SK1 and SK2 the intracellular N-terminal part of SK3 is extended and characterized by two glutamine repeats, one being

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highly polymorphic [6]. Channel activation by an intracellular rise in Ca^{2+} concentration is conferred through CaM that binds to the C-terminal part below the pore region. A model for SK channel gating was proposed based on biochemical data and information from the crystal structure of the SK channel CaMBD/ Ca^{2+}/ CaM complex [5, 7]. Upon Ca^{2+} binding the dimerization of CaM-CaMBD of the subunits follows, resulting in a rotary movement which is transmitted to the SK channel gate.

Since SK3 has a unique N-terminal part, distinguishing it from SK1 and SK2, we were interested, whether this difference might have some functional consequences. In order to extend information about N- and C-terminal parts of SK channels, we investigated N-terminal parts of SK1, SK2, and SK3 and the C-terminal part of SK3 by means of a LexA based yeast two hybrid system. In these experiments we found an interaction of the N-terminal part of SK3 with the N-terminal and the C-terminal part of SK3. N-terminal parts of SK1, and SK2 did not interact and there was no interaction among N-terminal parts of the SK channel members. In addition, we investigated a possible functional role of the N-C and N-N interactions of SK3. Therefore, we expressed amino acids 1-299 of SK3 in PC12 cells which contain endogenous SK3 channels. In these experiments 1-EBIO pharmacology but not Ca^{2+} sensitivity of endogenous SK3 channels was modified by amino acids 1-299 of SK3. Considering the results from yeast, 1-EBIO sensitivity of endogenous SK3 channels in PC12 cells was possibly changed by N-C interactions with amino acids 1-299 of SK3. We conclude from these results, that N-C interactions in SK3 are possibly important for a functional conformation of the channel function.

Materials and Methods

Cloning

PCR was used to generate constructs coding for in-frame fusions of cytoplasmic N- and C-terminal parts of SK1 (KCCN1), SK2 (KCCN2) and SK3 (KCCN3) with LexA of the yeast two hybrid vectors pLexA (*$LexA_{(1-202)}$ his3 Amp^r)* [8] (BD Biosciences Clontech, Palo Alto, CA). As templates SK1 (ac: U69883) and SK2 (ac: U69882) coding plasmids generously provided by Dr. John. P. Adelman, Vollum Institute, Oregon Health & Science University, Portland, USA were used [9]. The SK3 coding plasmids (ac: AF031185, and ac: AJ251016) were generously provided by Dr. K. George Chandy UCI, Irvine, USA. The following plasmids coding for fusion proteins SK1N_125-LexA, SK2N_136-LexA, SK3N_165-LexA, SK3N_274-LexA, and SK3N_299-LexA were constructed. The pB42AD derived plasmids were generated by subcloning *Eco*RI-*Xho*I fragments of pLexA-derived plasmids into pB42AD (acidic activator B42 *trp1* Amp^r) [8] (BD Biosciences Clontech, Palo Alto, CA) generating plasmids coding for SK1N_125-B42AD, SK2N_136-B42AD, SK3N_299-B42AD, fusion proteins. SK3C-B42AD and SK3C-LexA were designed by PCR containing amino acids 549-736 of SK3 (ac: AJ251016). All constructs generated by PCR were controlled by sequencing. Non of the constructs showed auto-induction in yeast with the exception of SK3C-LexA.

Corresponding to SK3N_299-LexA, several further constructs, coding for a different length in the polymorphic glutamine repeat, were generated by PCR amplification of the AAD14 sequence of SK3 [10] from patient DNA (AAD14 DNA was a generous gift from Dr. Deborah J. Morris-Rosendahl, University of Freiburg, Freiburg, Germany). For the expression in mammalian cells the coding region corresponding to amino acid 1 to 299 of SK3 (SK3N_299) was artificially placed in a Kozak consensus sequence of the vector pcDNA3.1 (pSK3N_299) (Invitrogen, Karlsruhe, Germany) using PCR-amplification. All constructs generated by PCR were verified by sequencing. The entire coding region of SK3 in pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA) (pEGFP-N1 SK3) which was used for confocal images was a generous gift from Dr. Oliver H. Wittekindt, University of Ulm, Germany [11].

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 and 10 % FCS for tsA201 cells (a HEK cell line stably transfected with the SV40 T-antigen) [12, 13]. For immunostainings and confocal images tsA201 cells were transfected with 2.5 ng pEGFP-N1 SK3 or with 2.5 µg pSK3N_299 using FUGENE 6 (Roche Applied Science, Mannheim, Germany) according to the manufacturers' protocol. 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 amino acids 1-299 of SK3 (SK3N_299), PC12 cells were transfected with a mixture of 2.4 µg pSK3N_299 and 0.1 µg of pEGFP-N1. PC12 cells were transfected using MATra (IBA GmbH, Goettingen, 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 electrophysiological measurements.

Yeast two hybrid experiments

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 [14]. The yeast reporter strain EGY48c [MATα, *his3, trp1, ura3, $lexA_{op(x6)}$ – LEU2*] [15] was transformed with the reporter plasmid p8op*lacZ* (*$lexA_{op(x8)}$ ura3* Amp^r) (Clontech, Heidelberg, Germany) and subsequently transformed with pLexA derived bait plasmids and pB42AD derived prey plasmid. p53-LexA (murine

p53) and T-B42AD (SV40 large T-Antigen) were used as positive controls [16, 17]. Transformants were tested for activation of reporter genes by growth on SD minimal plates with and without leucin 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 Leu and was judged by the development of blue color (beta gal activity). For a test of autoinduction strains were grown on glucose containing plates. Under these conditions the bait protein fusions are not made because the B42AD gene is under the GAL1 promotor control that is repressed by glucose and induced by galactose.

Patch-clamp experiments

Patch-clamp experiments were done with the whole cell recording mode of the patch clamp technique as decribed previously [18, 19]. For the measurements in Fig. 4 the internal 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. For the measurements with 1-EBIO in Fig. 6 internal solution was adjusted to 0.5 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. For the K aspartate solution we used instead of Na aspartate 160 mM K apartate. 1-EBIO (Tocris Cookson, Bristol, UK) was dissolved in DMSO and supplied as dilutions in K aspartate solution with 0.2, 0.3, 0.5, and 1 mM EBIO. In Fig. 4 and Fig. 6 current amplitudes in Na-solution at -80 mV were assumed to be background current and subtracted from the current amplitudes at -80 mV measured in K-solution.

The Ca^{2+} measurements were done in the whole-cell mode of the patch-clamp technique as described previously [18, 19], combined with fura-2 (Invitrogen, Karlsruhe, Germany) measurements. 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 µM $CaCl_2$ corresponding to 3 µM free Ca^{2+}, pH 7.2). PC12 cells contain endogenous Kir channels. For the electrophysiological measurements in Fig. 4 and 6, measurements were started after washout of the Kir channel, following dialyses of the cell contents within a few minutes [20]. For measurements of Ca^{2+} activation this was not possible because tip and pipette solutions mixed in between 10 min. Since Cs^+ was described to block Kir channels [20], we measured SK3 conductance in Cs^+ aspartate solution. As described before, SK3 can carry a significant Cs^+ current [21]. The Cs^+ aspartate solution contained (mM): 165 Cs aspartate, 2 $CaCl_2$, 1 $MgCl_2$ and 5 HEPES, pH 7.4. All values presented here are mean ± S.D with n the number of independent observations.

Measuring intracellular Ca^{2+}

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} 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 1.135 µM, 0.18, and 0.74.

Immunostaining

For immunostaining tsA201 cells were plated on poly-lysine coated glass cover slips. Afterwards they were fixed with 4 % paraformaldehyde for 15 min and washed 3 x with Dulbecco's phosphate buffered saline (DPBS). Permeabilization was done with 0.05 % saponine in DPBS for 30 min. Subsequent blocking was done in 1 % BSA for 1 hour. Next, cells were incubated for 1 h at 4°C with an anti-SK3-specific antibody which reacts with the N-terminus of SK3 (rabbit anti-SK3 $antibody_{2-21}$,Alamone Labs, Jerusalem, Israel) in carrier solution containing 1 % BSA in DPBS. Following washing 3 x in DPBS, cells were incubated with Alexa-Fluor® 488 goat anti-rabbit IgG (H+L) (Invitrogen, Karlsruhe, Germany) for 1 h at room temperature. Cells were mounted with Fluorescent Mounting Medium (DakoCytomation, Hamburg, Germany) and stored in the dark.

Confocal microscopy

Confocal pictures of immunostainings were done as described previously [11].

Results

The SK3 channel is characterized by a long N-terminal part that is twice as long compared to SK1 and SK2. In order to find out whether this long N-terminal part of SK3 has any physiological function, we investigated the interaction of N-terminal and C-terminal parts of SK3 with N-terminal parts of SK1, SK2, and SK3 using a LexA based yeast two hybrid system.

Yeast two hybrid experiments

Fig. 1 shows fusion proteins of the SK channels we used for our experiments. The N-terminal part of SK3, ending before S1 (SKCa3-1/285) exhibited a dominant negative effect on SK2 currents in Jurkat cells [22]. Therefore we used for SK1 and SK2 fusionproteins containing the entire N-terminal part predicted to be in

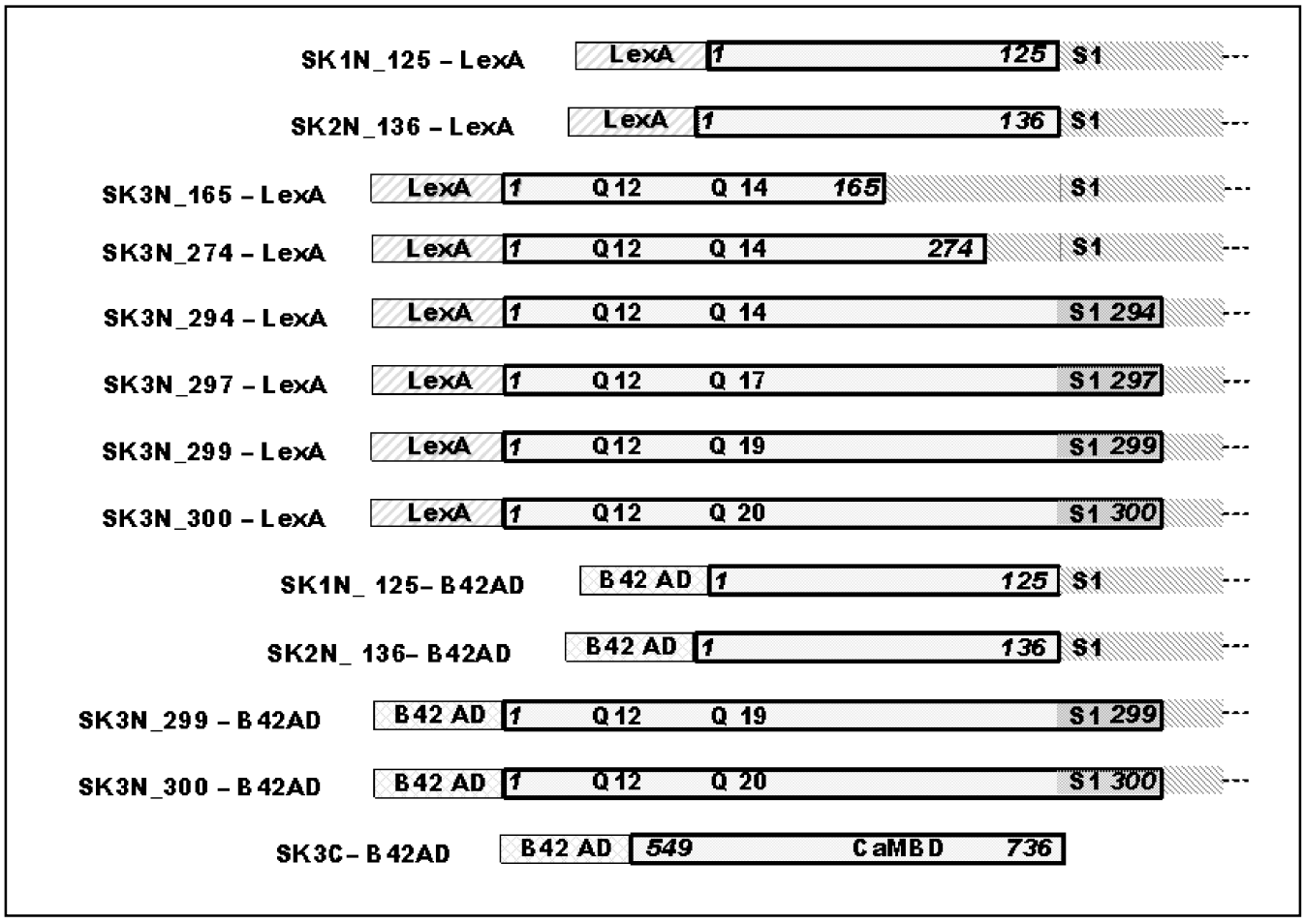

Fig. 1. Summary of constructs which were used in a LexA based yeast two hybrid system. Amino acid sequences of SK1, SK2, and SK3 were fused to a LexA DNA binding domain or a B42 AD transcription activation domain. Numbers of amino acids of SK channel sequences are in italics. Q: glutamine repeats, CaMBD: calmodulin binding domain.

the cytoplasm (see Fig. 1). In addition we made truncations of the cytoplasmic N-terminal part to 274 and to 165 amino acids and used an N-terminal fragment (aa 1-299) containing almost half of S1. We controlled stability of SK1, SK2 and SK3 fusion proteins by Western blotting (data not shown). All yeast transformants were streaked on selection plates shown in Fig. 2.

Interactions of SK channels found in yeast.

In yeast we observed an interaction between N- and C-terminal parts and between N-terminal parts of SK3 (SK3N_299-LexA interacted with SK3C-B42AD and SK3N_299-B42AD) (Fig. 1, Table 1). In contrast SK1N_125 and SK2N_136 fusion proteins did not interact with themselves and there was no interaction among N-terminal parts of SK1, SK2, and SK3 fusion proteins (Table 1A). Therefore the strength of the N-N interaction of SK3 might be unique in the SK channel family. Truncations of the N-terminal part of SK3 (SK3_274 and SK3_165) abolished interaction with SK3N_299 (Table 1B). Therefore the additional 25 amino acids of SK3N_299-LexA compared to SK3N_274-LexA revealed a functional conformation in yeast.

The SK3 N-tail contains two polyglutamine-repeats, one being highly polymorphic [6]. The number of glutamine repeats might influence the strength of the N-C and N-N interactions. In order to find this out, we generated SK3 N-terminal LexA and B42 AD fusions with 14, 17, 19, and 20 glutamine repeats and tested them for an interaction in yeast. All different N-terminal constructs of SK3 interacted and no major influence of the length of the glutamine repeat on the strength of SK3 N-C and N-N

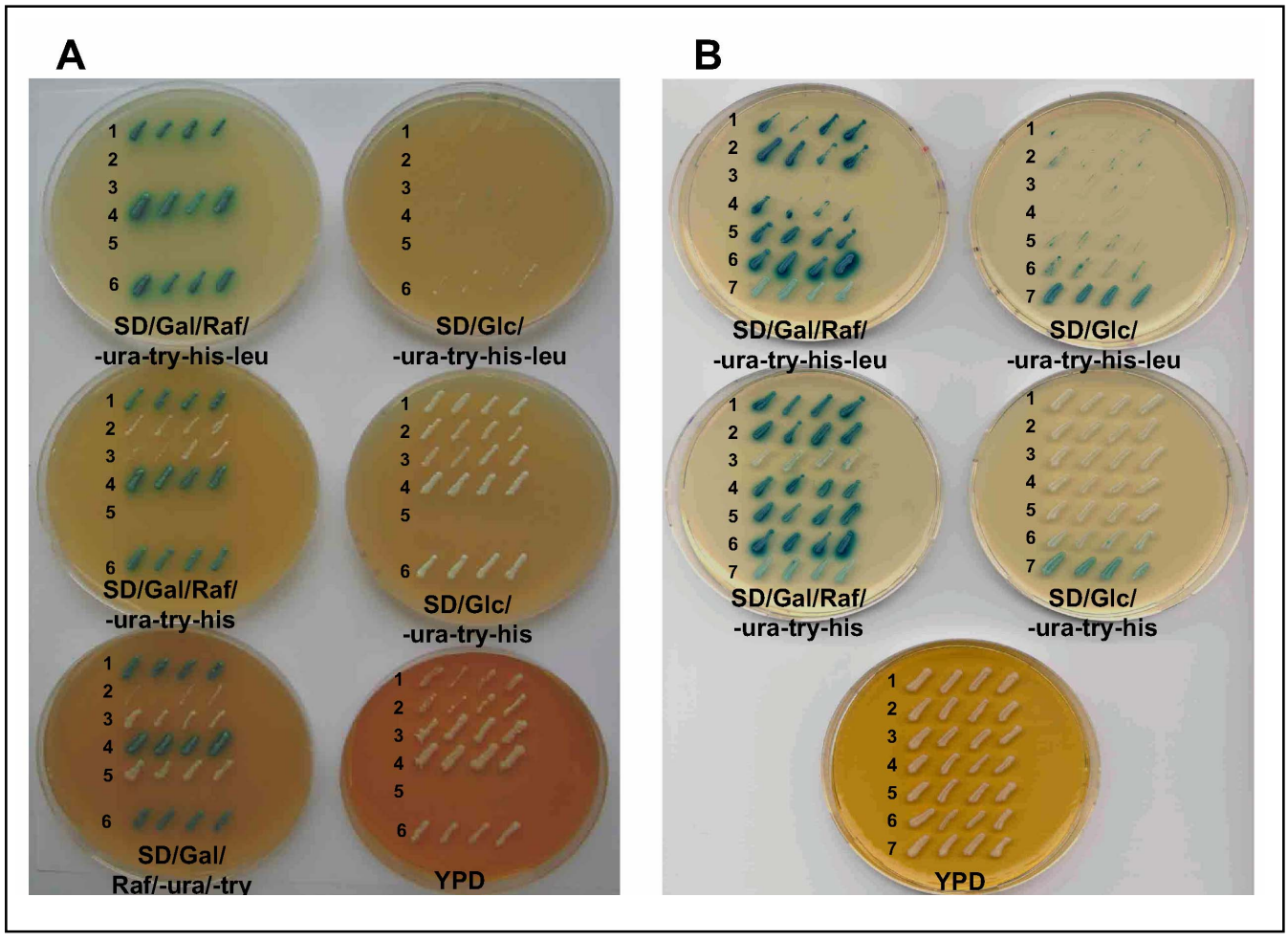

Fig. 2. Interaction of N-terminal parts and of the N-terminal with the C-terminal part of SK3. Of each transformation 4 individual colonies were streaked on SD/Glc/-Ura/-Try/-His/X-Gal/BU-salts-, SD/Glc/-Ura/-Try/-His/-Leu/X-Gal/BU-salts, SD/Gal/Raf/-Ura/-Try/-His/X-Gal/BU-salts-, SD/Gal/Raf/-Ura/-Try/-His/-Leu/X-Gal/BU-salts-minimal plates and on YPD plates as a growth control. A) Row 1: SK3N_299-LexA, (Q19)/ SK3N_299-B42AD, (Q19); Row 2: SK3N_165-LexA, (Q14)/ SK3N_299-B42AD, (Q19); Row 3: SK3N_274-LexA, (Q14)/ SK3N_299-B42AD, (Q19); Row 4: SK3N_299-LexA, (Q19)/ SK3C-B42AD; Row 5: SK3_C-B42AD; Row 6: p53-LexA/ T-B42AD. B) Row 1: SK3N_294-LexA, (Q14)/ SK3N_294-B42AD, (Q14); Row 2: SK3N_294-LexA, (Q14)/ SK3N_299-B42AD, (Q19); Row 3: SK3N_274-LexA, (Q14)/ SK3N_299-B42AD, (Q19); Row 4: SK3N_297-LexA, (Q17)/ SK3N_299-B42AD, (Q19); Row 5: SK3N_299-LexA, (Q19)/ SK3N_299-B42AD, (Q19); Row 6: p53-LexA/ T-B42AD; Row 7: "false positive", proteins which show unspecific growth and color development.

interaction could be observed in yeast (Table 1B and C).

Intracellular localization of SK3N_299

For the further analysis of a possible functional role of N-C and N-N interactions of SK3, we constructed a mammalian expression vector coding for amino acids 1-299 of SK3. Since it was reported that an N-terminal fragment of SK3 (SKCa3-1/285) with a similar length like SK3N_299 trafficked to the nucleus [22], we had to check whether SK3N_299 also accumulates in the nucleus or whether it is found in the cytoplasm where it would be available for an interaction with SK3 channels in further experiments. SK3N_299 was found in the cytoplasm and did not accumulate in the nucleus (Fig. 3B). Like expected the GFP-tagged SK3 channel was in the membrane and did also not accumulate in the nucleus (Fig. 3A) as had been described in the literature [11]. Untransfected tsA201 cells did not show any staining for SK3 protein (Fig. 3C) at all. These experiments showed that SK3N_299 was distributed in the cytoplasm and available for further investigations of a possible role of N-C or N-N interactions in SK3 channels.

A

	SK1N_125-B42 AD	SK2N_136-B42 AD	SK3N_299-B42 AD (Q19)	SK3C-B42 AD
SK1N_125 -LexA	-	-	-	/
SK2N_136 -LexA	-	-	-	/
SK3N_299 -LexA (Q19)	/	/	+	+

B

	SK3N_299-B42 AD
SK3N_165 -LexA (Q14)	-
SK3N_274 -LexA (Q14)	-
SK3N_294 -LexA (Q14)	+
SK3N_299 -LexA (Q19)	+

C

	SK3N_299-B42 AD (Q19)	SK3N_300-B42 AD (Q20)	SK3C-B42 AD
SK3N_294 -LexA (Q14)	+	+	+
SK3N_297 -LexA (Q17)	+	+	/
SK3N_299 -LexA (Q19)	+	+	+
SK3N_300 -LexA (Q20)	/	+	+

Table 1. Summary of results of the yeast two hybrid experiments. + : interaction; - : no interaction; / : not determined.

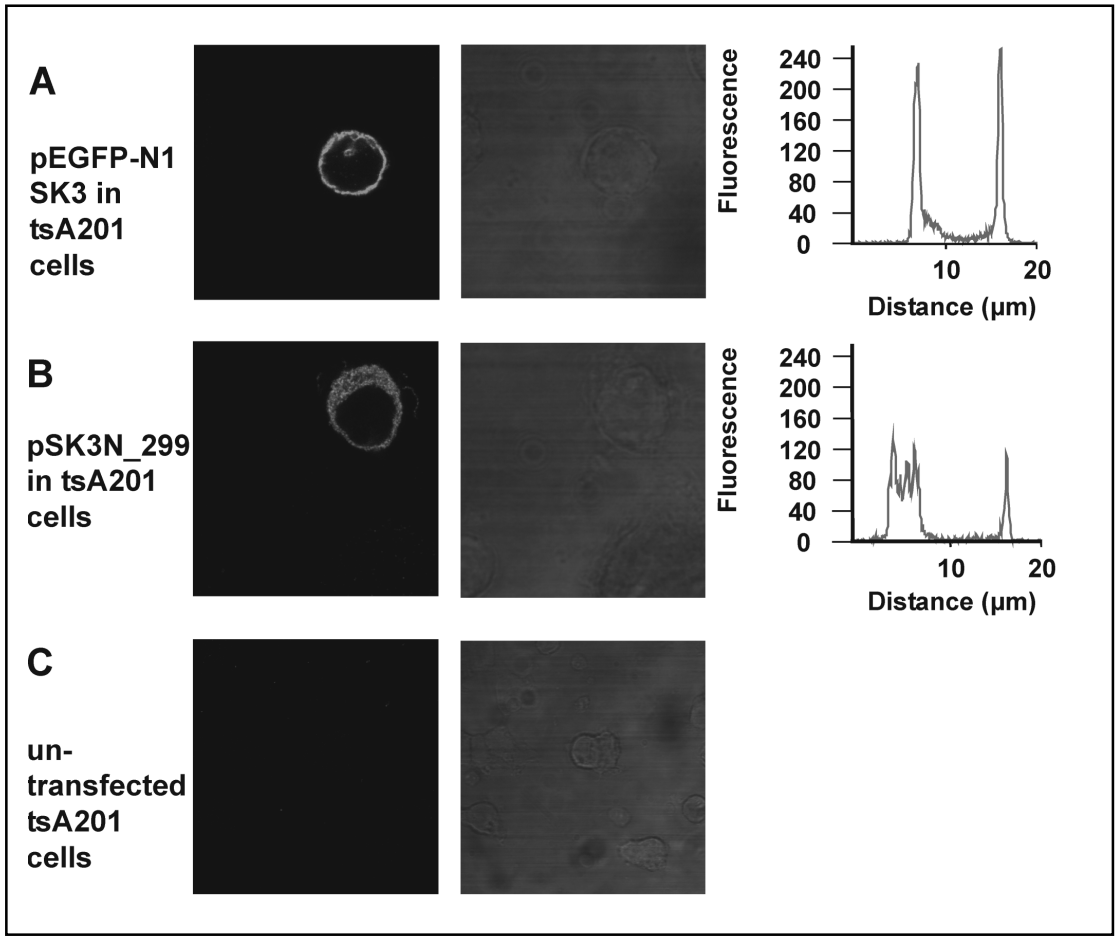

Fig. 3. Confocal and transmission light images of tsA201 cells after immunolabeling with an anti-SK3 antibody and the corresponding intensity histograms. (A) tsA201 cells expressing SK3, (B) tsA201 cells expressing SK3N_299, (C) no SK3 staining in untransfected tsA201 cells immunolabeled with anti-SK3 antibody. The confocal images and the corresponding intensity histograms for fluorescence show neither in (A) nor in (B) an accumulation of fluorescence in the nucleus.

What is the function of the interactions of cytoplasmic parts in SK3?

For the investigation of a possible role of the N-C or N-N interactions, we expressed amino acids 1-299 of SK3 (SK3N_299) in PC12 cells, which contain endogenous SK3 channels [23, 24]. For control experiments we transfected PC12 cells with pEGFP-N1 to exclude a non-specific effect of the vector on the expression of endogenous SK3 channel.

First of all, we examined by whole cell measurements if SK3N_299 might assemble with full length SK3 subunits and reduce the number of functional SK3 channels in the membrane. Fig. 4A shows a representative SK3 current-amplitude of control transfected PC12 cells.

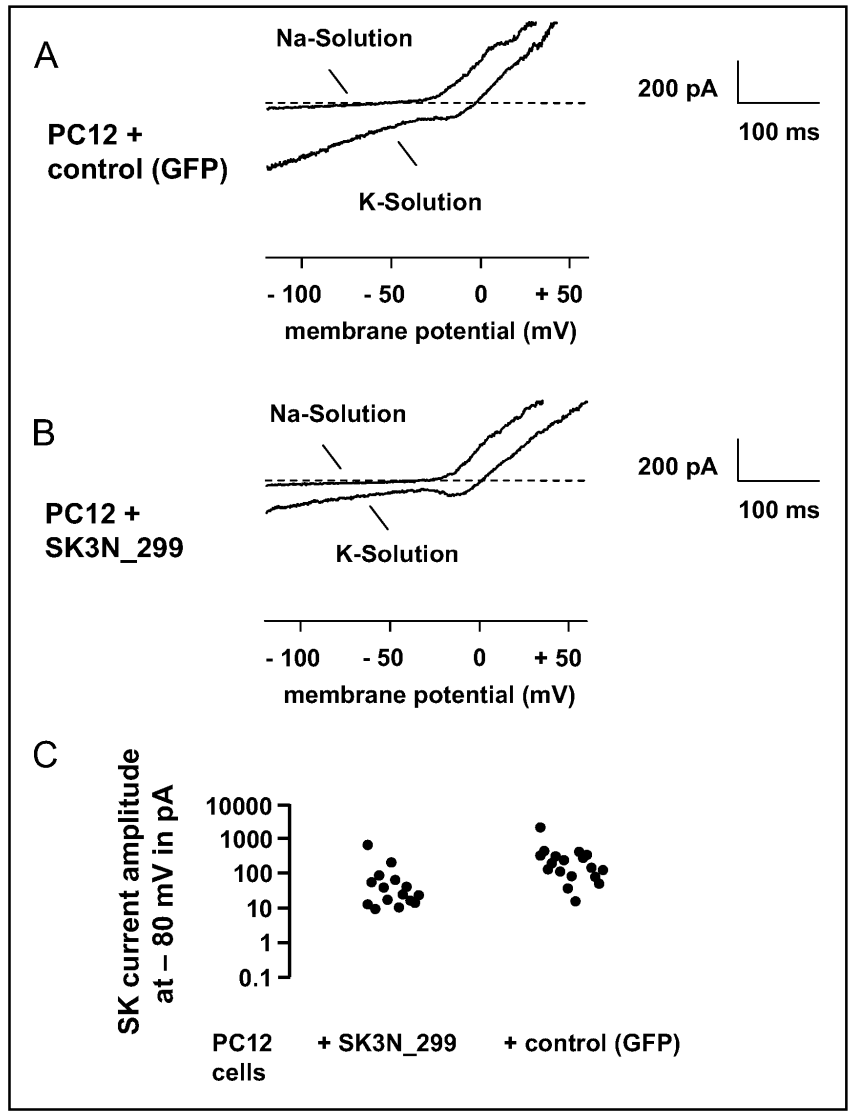

Fig. 4. Current amplitudes of endogenous SK3 currents in PC12 cells expressing SK3N_299. (A), Representative endogenous SK3 current in control (pEGFP-N1) transfected cells. (B), Representative endogenous SK3 currents in cells transfected with pSK3N_299 (C), Scatter plot showing current amplitudes of control (GFP) and SK3N_299 expressing PC12 cells at -80 mV.

At -80 mV current amplitude in Na-solution was -14 pA and increased in K-solution to -211 pA (Fig. 4A). PC12 cells transfected with pSK3N_299 showed in Na-solution at -80 mV a current-amplitude of –11 pA which increased in K-solution to –95 pA (Fig. 4B). The scatter plot in Fig. 4C summarizes the obtained data. SK3 current amplitudes of SK3N_299 expressing cells were found to be not different from control expressing cells by applying a two-tailed Student's t test ($p = 0.08$). Therefore, SK3N_299 had no influence on the number of endogenous SK3 channels functional in the membrane of PC12 cells.

Do N-C interactions influence Ca^{2+} sensitivity of SK3 channels?

The number of functional channels was not changed by SK3N_299. The next question, which had to be answered, was whether SK3N_299 changes function of endogenous SK3 channels in PC12 cells. One could imagine that SK3N_299 might interact - like the N-terminal part of the full-length channel - with the C-terminal part of the channel which confers the channel activation through Ca^{2+} [4, 5]. Therefore, we examined if SK3N_299 changes Ca^{2+} activation of SK3 channels. We measured whole-cell conductance (Fig. 5A and 5B) and in parallel the internal Ca^{2+} concentration. After subtraction of the intensity of the auto-fluorescent light, ratios for F_{350} and F_{380} of the fura measurements were used to calculate the Ca^{2+} concentration according to the equation stated in material and methods. For further data analyses simultaneously determined whole cell conductances were plotted against the Ca^{2+} concentration for each experiment, separately. Curves were fitted according to the equation $g/g_{max} = 1/(1 + (EC_{50} / [Ca^{2+}])^{nH})$, where g_{max} is the maximal conductance, EC_{50}

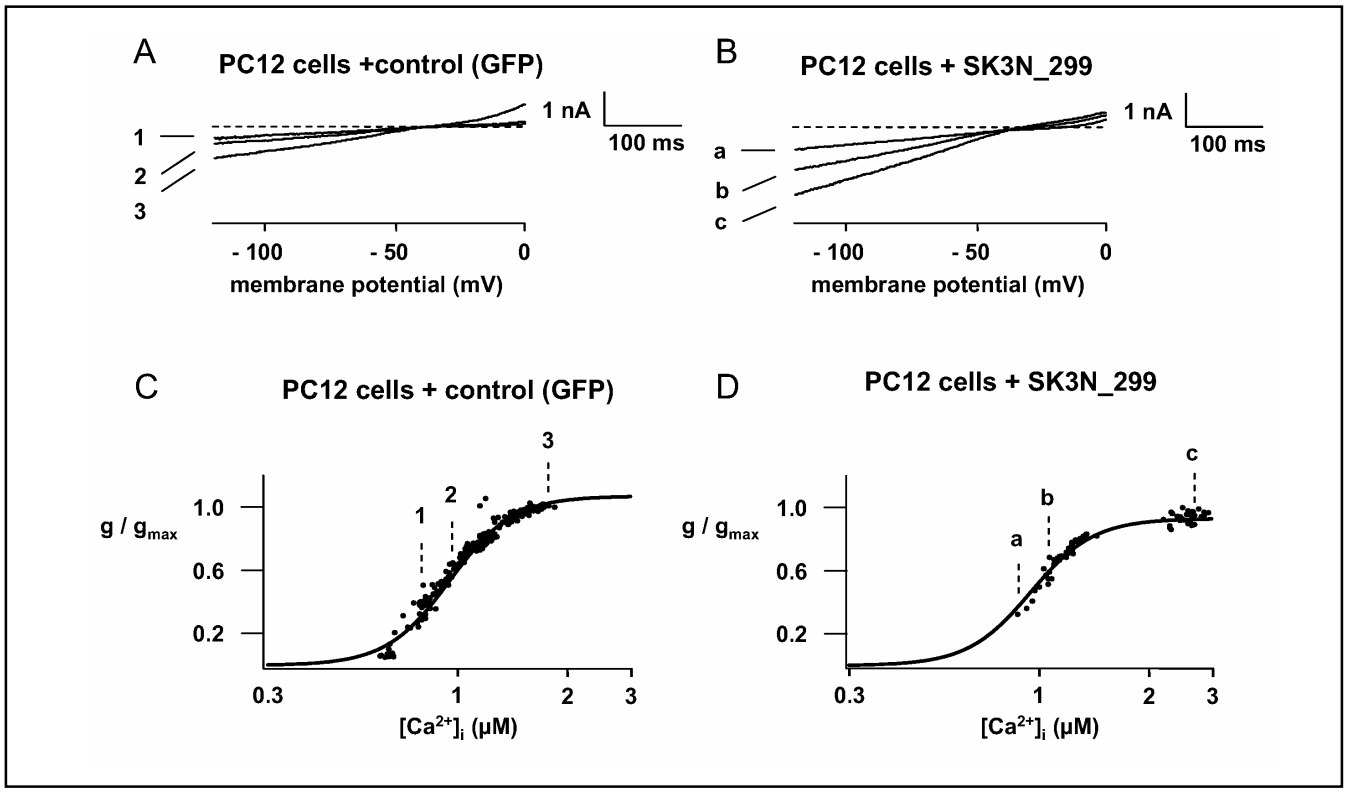

Fig. 5. Activation of SK3 channels in PC12 cells expressing SK3N_299 or a control (GFP) by internal Ca^{2+}. Membrane potentials were clamped from -120 to +60 mV in 400 ms. In parallel, internal Ca^{2+}concentration was measured by the fura-2 method. Cs^{+}-aspartate solution was used as external solution. 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 SK3 current in control transfected cells. (B), Representative endogenous SK3 current in cells transfected with pSK3N_299. (C), and (D), for calculation of the EC_{50} values for Ca^{2+}, whole-cell conductances were calculated for the interval from – 100 to - 50 mV and were plotted against the Ca^{2+} concentration measured at the same time. Curves were fitted by eye according to the equation $g/g_{max} = 1/(1 + (EC_{50} / [Ca^{2+}])^{nH})$, where g_{max} is the maximal conductance, EC_{50} is the half-maximal activating Ca^{2+} concentration, and n_H is the Hill coefficient. The EC_{50} and the Hill coefficient were calculated for control (GFP) and SK3N_299 expressing cells as the mean from three independent experiments. In control expressing cells the EC_{50} was 1 ± 0.1 µM and the Hill coefficient 5.7 ± 1.2. For SK3N_299 expressing cells the EC_{50} was 1 ± 0.1 µM and the Hill coefficient 4.8 ± 0.8.

is the half-maximal activating Ca^{2+} concentration, and n_H is the Hill coefficient (Fig. 5, C and D). The results showed that the Ca^{2+} sensitivity of SK3N_299 expressing cells (EC_{50} = 1 ± 0.1 µM, Hill coefficient = 4.8 ± 0.8, n=3) was not different in comparison to control expressing cells (EC_{50} = 1 ± 0.1 µM, Hill coefficient = 5.7 ± 1.2, n=3). SK3N_299 had no effect on Ca^{2+} sensitivity of endogenous SK3 channel in PC12 cells. From these experiments we conclude that an interaction of SK3N_299 with the C-terminal part of endogenous SK3 channels conferring Ca^{2+} sensitivity did not change Ca^{2+} activation of SK3 channels.

N-C interactions of SK3 channels influence 1-EBIO sensitivity

Besides a change in Ca^{2+} sensitivity, one could also imagine a change in 1-EBIO sensitivity by N-C interactions of SK3N_299 with SK3 channels. The increase in Ca^{2+} sensitivity of SK channels by 1-EBIO is thought to be conferred by the C-terminal part of SK channels [25]. An N-C interaction might therefore change sensitivity by interfering with the 1-EBIO binding site or the activation mechanism. Therefore we expressed SK3N_299 in PC12 cells and investigated if there is an interference of SK3N_299 with the 1-EBIO binding of SK3 channels.

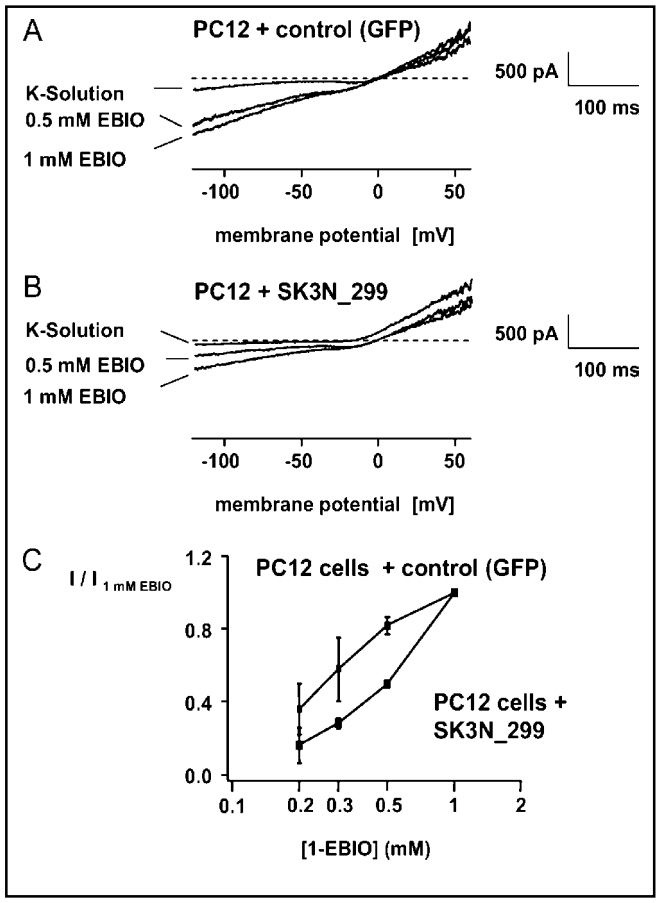

Fig. 6. Activation of endogenous SK3 currents with different 1-EBIO concentrations. (A) representative endogenous SK3 current in control (pEGFP-N1) transfected cells. (B) representative endogenous SK3 currents in cells transfected with pSK3N_299. (C) activation of SK3 current by different 1-EBIO concentrations (in comparison to the activation of SK3 current by 1 mM 1-EBIO) of control (GFP) and SK3N_299 expressing cells.

To elicit SK3 current, the pipette solution contained 0.5 µM Ca^{2+}. 1-EBIO concentrations of 0.2, 0.3, 0.5, and 1 mM were applied with the bath solution. Fig. 6A shows a representative ramp current trace through endogenous SK3 channels of a control transfected PC12 cell in K-solutions with different 1-EBIO concentrations. In K-solution without 1-EBIO current amplitude was -87 pA at -80 mV and raised to -392 pA in K-solution with 0.5 mM 1-EBIO and to -485 pA in K-solution with 1 mM 1-EBIO. In PC12 cells transfected with pSK3N_299 applying different 1-EBIO concentrations resulted also in an increase of current amplitude (Fig. 6B). In K-solution without 1-EBIO current amplitude at -80 mV was -41 pA and raised to -145 pA in K-solution with 0.5 mM 1-EBIO and to -255 pA in K-solution with 1 mM 1-EBIO. The diagram in Fig. 6C summarizes 1-EBIO activation of SK3 channels in control and in pSK3N_299 transfected PC12 cells. In control transfected PC12 cells 0.2 mM 1-EBIO activated 36 %, 0.3 mM 1-EBIO 58 % and 0.5 mM 1-EBIO 82 % of the channels which were activated with 1 mM 1-EBIO (n = 3). In pSK3N_299 transfected cells 0.2 mM 1-EBIO activated 14 %, 0.3 mM 1-EBIO 28 % and 0.5 mM 1-EBIO 50 % of the channels which were activated with 1 mM 1-EBIO (n = 3). SK3N_299 reduced 1-EBIO sensitivity of endogenous SK3 channels in PC12 cells. We conclude that SK3N_299 interacts with SK3 channels in PC12 cells thereby modifying the 1-EBIO binding.

Discussion

Yeast-two-Hybrid-Experiments

The results presented here show that the N-terminal part of SK3 channels interacted with the N-terminal and with the C-terminal part of SK3 in yeast.

There was no N-N interaction of SK1 and SK2, and the different SK channel members did not show interaction of N-terminal parts among each other. The strength of the N-N interaction of SK3 might therefore be unique in the SK channel family. If N-terminal parts of SK channels play a role in tetramerization like the N-terminus (T1 domain) in Kv channels, the comparatively strong interaction between N-terminal parts of SK3 could favour SK3 homotetramerization in tissues were all three members of the SK channel family are expressed. Truncation of the N-terminal part to a length of 274 amino acids (SK3N_274) and 165 amino acids (SK3N_165) abolished the interaction with SK3N_299 in yeast. Truncation of the N-terminal part of SK3 might delete amino acids which might disturb a functional conformation.

Intracellular localisation of amino acids 1-299 of SK3

Recently, a rare frame shift mutation leading to a premature stop codon at position 286 in SK3 was found in a schizophrenic patient [26]. The frameshift mutation was predicted to generate a truncated SK3 fragment containing the whole cytoplasmic N-terminal part and ending shortly before S1 (SKCa3-1/285, aa 1-283 of AF031815, predicted start of S1 in AF031815 is aa 285). Partly mediated by two nuclear localisation signals (NLS)

the truncated protein trafficked to the nucleus and suppressed endogenously expressed SK2 currents in Jurkat cells [22].

SK3N_299 did not accumulate in the nucleus, like it was reported for shorter constructs [22]. This observation supports the hypothesis that additional amino acids in S1 which are included in SK3N_299 might stabilize a functional conformation. This conformation could help to abrogate or shield the function of the NLS motifs in the SK3 N-terminal part, and therefore the SK3N_299 fragment is not imported to the nucleus. This conformation might also be responsible for a better accessibility of interacting amino acids in SK3N_299 and could account for a stronger interaction of SK3N_299 in comparison to shorter truncations of the SK3 N-terminal part (truncation to 274 and 165 amino acids) which did not interact any more in yeast.

N-C interactions in SK3

What could be the function of the N-C interactions of SK3 identified in yeast? N-C interactions in SK3 might modulate channel function via Ca^{2+}/ CaM binding to the CaMBD in the C-terminal part of each SK α–subunit.

Beside Ca^{2+}/ CaM binding, the binding of other interaction partners might be modulated by N-C interactions of SK3. 1-EBIO for instance is thought to interact with the SK channel α-subunit by stabilization of the interaction between Ca^{2+}-CaM and the SK channel α-subunit but the binding site is still unknown [25]. Pedarzani and colleagues (2001) showed that 1-EBIO had no effect on channel activation in the absence of $[Ca^{2+}]_i$ or with saturating $[Ca^{2+}]_i$ while at low $[Ca^{2+}]_i$ Ca^{2+} sensitivity of SK channels was increased. In further experiments with mutant CaMs and chimeric SK/IK channels they concluded that the C-terminal part of the SK channels determine 1-EBIO pharmacology. Corresponding to the N-C interactions which we identified in yeast, one could imagine that N-C interactions in SK3 channels possibly modulate the binding of 1-EBIO.

In order to investigate if N-C interactions of SK3 might have functional consequences, we measured Ca^{2+} and 1-EBIO sensitivity of endogenous SK3 channels in PC12 cells, expressing amino acids 1-299 (SK3N_299) of SK3. SK3N_299 might interact with endogenous SK3 channels in PC12 cells by N-C interactions and modulate Ca^{2+} or EBIO sensitivity. When we expressed amino acids 1-299 of SK3, 1-EBIO sensitivity of endogenous SK3 channels in PC12 cells was reduced. In contrast to the change in 1-EBIO sensitivity, Ca^{2+} activation was not modified. The simplest explanation for this result is to assume an interaction of SK3N_299 with the C-terminal part of endogenous SK3 channels, thereby modulating 1-EBIO pharmacology. We assume that the cytoplasmic N-tail SK3N_299 - possibly not anchored in the membrane - may bind the full-length channels thereby modifying the physiological binding sites for the N-terminal segment of the full-length channel. Another possibility would be that SK3N_299 competed with inter- or intra-subunit N-C-interactions of SK3 channels which might be important for channel function. Taken together, we conclude from these results that N-C interactions in SK3 channels are important for channel function. Similar conclusion had been reached from other channels (see below).

Examples for N-C interactions from the literature

N-C interactions can differently influence channel function. One of the examples about this comes from rat olfactory CNG channels. Varnum and Zagotta (1997) identified N-C interactions in these channels *in vitro*, and the N-C interactions were blocked by Ca^{2+}/ CaM [27]. An N-terminal deletion disrupted the N-C interaction *in vitro* and reduced sensitivity to cyclic nucleotides as well as to CaM of the expressed channels.

Another example comes from Kir1.1 (ROMK) channels. These channels are highly sensitive to the intracellular pH [28]. Rapedius and colleagues (2006) identified N-C intra-subunit and C-C inter-subunit interactions in these channels [29]. Mutations in the interacting residues affected the pH-sensitivity of Kir1.1 channels thereby demonstrating the importance of N-C and C-C interactions for the pH-sensitivity of Kir1.1 channels.

Conclusion

In yeast, we identified N-N and N-C interactions of SK3 tails. Expression of amino acids 1-299 of SK3 modulated 1-EBIO pharmacology of endogenous SK3 channels in PC12 cells. Considering the results from yeast, amino acids 1-299 of SK3 possibly interacted by N-C interactions with endogenous SK3 channels, thereby changing 1-EBIO pharmacology. From these results we conclude that N-C interactions occur in SK3, which might be important for the function of SK3 channels.

Abbreviations

SK channels (small conductance Ca^{2+} activated K^+ channels); SK1-SK3 (KCNN1-KCNN3), members of

the SK channel family; AHP (afterhyperpolarisation); AP (action potential); CaMBD (calmodulin binding domain); CaM (calmodulin); NLS (nuclear localisation signal); N-N interaction (interaction between cytoplasmic N-terminal parts); N-C interaction (interaction between the cytoplasmic N-terminal with the cytoplasmic C-terminal part); S1-S6 (putative transmembrane segments); Kv channels (voltage gated K channels); T1 (tetramerization domain in Kv channels); 1-EBIO (1-ethyl-2-benzimidazolinone); 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); IK channels (intermediate conductance Ca^{2+} activated K channels (KCNN4)).

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