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Characterization of the effects of Endophilin A3 on the physiological properties of SK3 channels

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ABBREVIATIONS

1-EBIO	1-ethyl-2-benzimidazolinone	EGTA	ethylene glycol
2YT	double yeast extract tryptone medium	EC_{50}	half maximal activating concentration
A	alanine	FBS	fetal bovine serum
Abi-1	Abelson interacting protein 1	HEPES	4-(2-hydroxyethyl)-1-piperazine-ethene sulfonic acid
AHP	afterhyperpolarization	HS	horse serum
Asp	aspartate	Htt	Huntingtin
°C	degree celcius	Iba1	ionized Ca^{2+} binding adapter molecule
Ca^{2+}	calcium ion	IC_{50}	half-maximal inhibitory concentration
$[Ca^{2+}]_i$	intracellular Ca^{2+}concentration	K	lysine
$CaCl_2$	calcium chloride	K^+	potassium ion
CaM	calmodulin	K_D	dissociation constant
CaMBD	calmodulin binding domain	K_{eff}	effective dissociation constant
CK2	protein kinase 2	L	leucine
Cs^+	cesium ion	LB	lysogeny broth
DA SN	dopaminergic neurons in the substantia nigra	M	methionine
DC-EBIO	5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one	mAHP	medium afterhyperpolarization
EGFR	epidermal growth Factor Receptor	MEM	minimal essential medium Earl's salts

$MgCl_2$	magnesium chloride	PP2A	protein phosphatase 2A
min	minute	pM	picomolar
µl	microliter	pS	picosiemens
ml	milliliter	Q	glutamine
mm	millimeter	R	arginine
mV	millivolt	rpm	rounds per minute
Na^+	sodium ion	RT	room temperature
NaCl	sodium chloride	s	second
nm	nanometer	SD	standard deviation
nM	nanomolar	SH3	Src-homology-3 domain
NS308	6,7-dichloro-1 hindole-2,3-dione 3-oxime	SH3GL	SH3-containing GRB2-like
nWASP	neural Wiskott Aldrich syndrome protein	STIM 1	stromal interaction molecule 1
OD	optical density	T	threonine
Orai-1	calcium release-activated calcium channel	TM	transmembrane
P	pore helix	TRPV4	transient receptor potential cation channel subfamily V member 4
pA	picoampere	UCL 1848	bis-quinolinium cyclophane
PBS	phosphate buffered saline without calcium		

1 INTRODUCTION

Ion channels are integral membrane proteins spanning the lipid bilayer creating conducting pores. They are present in the plasma membrane and intracellular organelles of almost all living cells (eukaryotic and prokaryotic cells), allowing the passage of ions in and out of the cell. Ion channels are characterized by their selective permeability and their gating mechanism (Hille, 2001). Based on the gating mechanism, ion channels can be divided into: voltage-gated ion channels, ligand-gated ion channels (also known as ionotropic receptors), light-gated ion channels, mechanosensitive ion channels, cyclic nucleotide-gated ion channels, and calcium-gated ion channels. Another classification is based on the type of the conducted ion, there are potassium, calcium, sodium, chloride, proton and non-selective ion channels (Guyton and Hall, 2006).

All experiments in my work were performed on SK3 channels which are members of the small-conductance calcium-activated potassium channel subfamily. Therefore, in the next paragraphs I will talk shortly about potassium channels in general and then about calcium-activated potassium channels.

1.1 Potassium channels

Potassium channels are found in a vast variety of tissues and cells including both excitable and non-excitable cells and healthy and tumor cells (Littleton and Ganetzky, 2000;Hille, 2001). Potassium channels are responsible for the transport of potassium ions through the hydrophobic membrane. They play a central role in the maintaining of different physiological processes such as muscle contraction, nerve excitation, regulation of cell volume, hormone and neurotransmitter secretion, cell proliferation, cell differentiation and cell morphogenesis (Jäger et al., 2004; Tharp et al., 2006; Liebau et al., 2007; Wang et al. 2008). In excitable cells, they act to set or reset the resting membrane potential. They play also an important role in sensory transduction, modulation of blood pressure, stress response, cognitive functions, metabolic regulation, fertilization and apoptosis (Ashcroft, 2006). In addition, they are important for the mitogenesis (Decoursey et al., 1984), for proliferation of melanoma cell (Nilius and Wohlrab, 1992), breast carcinoma cells (Strobl

et al., 1995), fibroblasts (Rane, 1999), and Schwann cells (Pappas and Ritchie, 1998). Thus, malfunction of these ion channels may lead to serious diseases, such in arrhythmias as they contribute to the regulation of the action potential duration.

The pore-forming subunits of potassium channels are encoded by more than 100 genes. Based on the number of the transmembrane helices and the P-loop, potassium channels can be divided into four main classes (see also Fig. 1): a) 2TM/P channels, which consist of two transmembrane (TM) helices with a P loop between them, exemplified by inwardly rectifying K^+ channels and by bacterial K^+ channels such as *KcsA*. b) 4TM/2P channels, which consist of two repeats of 2TM/P channels (Doyle et al., 1998; Jiang et al., 2003). c) 6TM/P channels, consist of six transmembrane helices and a P loop between the fifth and the sixth TM. d) 8TM/2P channels, which are hybrids of 6TM/P and 2TM/P, and were first found in yeast.

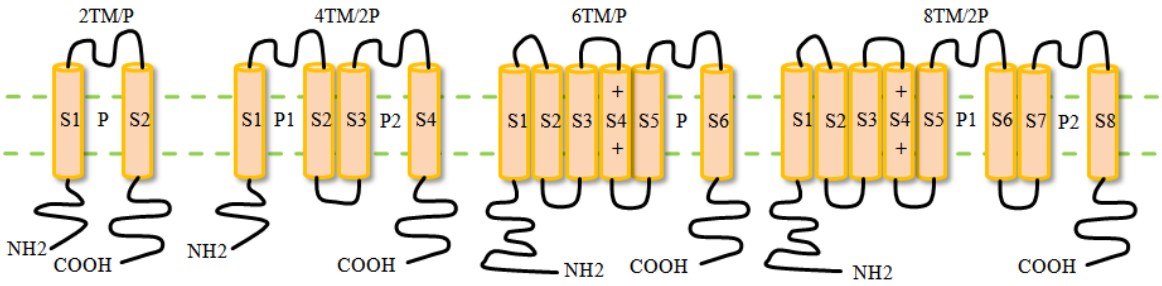


Fig. 1: Schematic classification of potassium channel subunits. The main four classes of potassium channels according to the structural characteristics of their subunits.

Structurally, potassium channels exist as tetramers consisting of the assembly of 4 α–subunits forming the transmembrane aqueous pore through which K^+ specifically flow. X-Ray analysis (Doyle et al., 1998; Jiang et al., 2003) revealed that the four subunits make an inverted teepee or cone holding the selectivity filter of the pore in its outer end (Fig. 2).

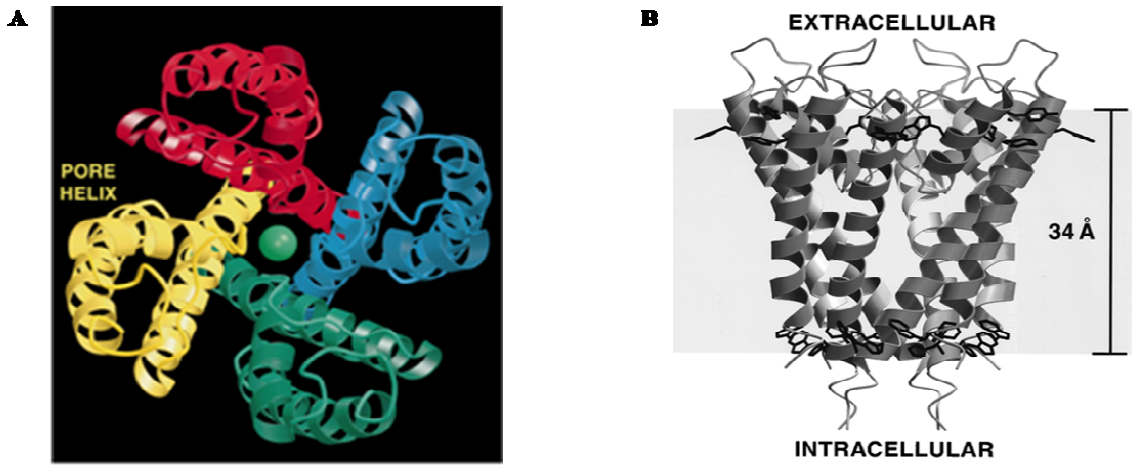


Fig. 2: A) Ribbon representation illustrating the three-dimensional fold of the *KcsA* (*Streptomyces lividans* potassium channels) tetramer viewed from the extracellular side and showing the pore helices. **B)** Ribbon representation of the tetramer as an integral membrane protein. This side view reveals the inverted-teepee shape of the channel. The channel is shown embedded in a cell membrane (from Doyle et al., 1998. P73).

1.2 Calcium-activated potassium channels (K_{Ca} channels)

Ca^{2+}-activated potassium channels (K_{Ca} channels) belong to the 6TM/P channel class. The name of these channels indicates the role of calcium in their activation. Therefore I would like first to mention briefly the role of intracellular Ca^{2+}before describing the K_{Ca} channels. Calcium ions exist in cells in different concentrations and are fundamental in several cell functions. They play an important role in many physiological processes such as signal transduction, neurotransmitter release, and muscle cell contraction. In a typical cell, the cytosolic calcium concentration at rest is kept at low levels about 100 nM, whereas the extracellular concentration is in the millimolar range around 1,2 mM. That low intracellular calcium concentration is maintained through internal Ca^{2+}-stores and various membrane calcium pumps. Responding to several signals and during various cellular functions, the intracellular calcium concentration may increase to values 10- to 100-fold higher than the normal value. This increase in the intracellular Ca^{2+} concentration may activate, i.e. open Ca^{2+}-activated K^+ channels (K_{Ca} channels). First indications about the activation of ion channels upon an increased cytosolic Ca^{2+} concentration were identified in red blood cells by Gardos (1958). Once the cytosolic Ca^{2+} concentration rises to micromolar ranges, K_{Ca} channels open and allow the passage of K^+ down their electrochemical gradient. K_{Ca} channels differ in their molecular and pharmacological

properties. Based on their conductance, K_{Ca} channels are divided into three major groups: big-conductance (BK) channels with conductances in the range of 200-400 pS, intermediate-conductance (IK) channels with 20–100 pS and small-conductance (SK) channels with 2-20 pS.

In the following paragraphs, I will write about SK channels in general and then especially about SK3 channels because my experiments were performed on these channels.

1.2.1 Small-conductance calcium-activated potassium channels (SK)

The cloning of the genes encoding the SK channels uncovered three genes SK1, SK2 and SK3 ($K_{Ca}2.1$, $K_{Ca}2.2$, $K_{Ca}2.3$) belonging to the KCNN gene family. The structural features of SK channels are similar to those of the voltage-gated potassium channels (K_v channels) in that they belong to the 6TM/P loop category. Each subunit is made up of six transmembrane (TM) hydrophobic alpha helices domains (S1 – S6) with cytosolic N- and C-terminal parts (Stocker, 2004). The permeation module of the channel is formed by the segments S5 and S6 plus the P-loop between the S5 and S6. The S4 segment in K_v channels has positively charged residues mediating thereby the voltage sensitivity of the channels. However, SK channels are activated in a voltage-independent manner despite the three positively charged residues in S4 (Blatz and Magleby, 1986; Köhler et al., 1996; Hirschberg et al., 1998).

SK subunits show an 80 to 90 % identity in their TM domains, however, they differ in the sequence and length of the N- and C-terminal parts (Köhler et al., 1996). That difference suggests that these domains are important to play distinct physiological roles and to mediate specific functions in response to intracellular signals. On the other hand, SK subunits show structural differences in the outer vestibule which determine sensitivity toward channel blockers (Ishii et al., 1997). In addition, these differences may reflect differential regulation by endogenous extracellular ligands.

Functional SK channels are formed by both homomeric (Chandy et al., 1998, Stocker and Pedarzani, 2000; Wolfart et al., 2001) and heteromeric subunit assembly (Stocker et al., 1999; Benton et al., 2003; Monaghan et al., 2004; Mongan et al., 2005; Strassmaier et al., 2005). However, the physiological importance of heteromeric channel assembly is not yet determined. Further diversity in the primary structure of the SK channels is generated by alternative exon splicing producing several isoforms: 32 isoforms for SK1 channels

(Schmuckler et al., 2001), two SK2 isoforms (Strassmaier et al., 2005), and three SK3 isoforms (Tomita et al., 2003; Wittekindt et al., 2004a; Wittekindt et al., 2004b). What is the physiological importance of these splice diversity? It is shown that some of these different splice variants have a negative effect on the normal channel isoform in that they prevent the transport of the normal channel to the plasma membrane reducing thereby the expression level of the functional channels. It seems that this complex transcription pattern may be involved in the tuning of the SK channels function, leading to gradations in the levels of membrane excitability (Tomita et al., 2003; Wittekindt et al., 2004a; Wittekindt et al., 2004b).

SK channels are predominantly expressed in the nervous system where they contribute to the afterhyperpolarization (AHP), especially the medium afterhyperpolarization (mAHP) which follows an action potential (Köhler et al., 1996), controlling thereby the synaptic plasticity and the intrinsic excitability of neurons (Chen and Toney, 2009). In addition, SK channels were also found in non-neuronal tissues such as human and mouse cardiac myocytes, predominantly in the atria and pace making tissues where they play an important role in the atrial repolarization and contribute to control the shape and the duration of cardiac action potentials (Tuteja et al., 2010).
As mentioned above, SK channels are not gated by changes in the membrane potential. So, how are they activated? In the next section, I will discuss the gating mechanism of SK channels.

1.2.2 Gating of SK channels

SK channels are not gated by voltage but by an increase in the intracellular calcium concentration (Blatz and Magleby, 1986; Hirschberg et al., 1998). The half maximal activation of SK channels was found in the range of 400 to 800 nM indicating their high Ca^{2+}-sensitivity (Park, 1994) with a K_D value around 0.3 µM (Xia et al., 1998). Unlike K_v channels, SK channels do not desensitize, their opening is not affected by prolonged exposure to Ca^{2+} (Hirschberg et al., 1998). Although SK channels are high-affinity calcium sensors, studying their gating mechanism revealed that calcium ions did not bind directly to the channel subunit, rather they bind to an EF-hand calcium-binding protein, calmodulin (CaM). How does CaM transmit the signal to the channel after Ca^{2+}-binding? Xia et al., (1998) showed that functional SK channels are heteromeric complexes with

calmodulin (Fig. 3) which is constitutively bound to a highly conserved, CaM-binding domain (CaMBD) in the membrane-proximal region of the cytosolic C-terminus of each subunit (Xia et al., 1998). That CaMBD is located immediately adjacent to S6 such that structural changes within or between CaMBD molecules might be directly transmitted to elicit gating (Schuhmacher et al., 2001). A functional SK channel binds at least 4 CaM (Fig. 3A).

Bound CaM consists of N- and C-lobe EF hands (Xia et al., 1998). CaMBD/Ca^{2+}/CaM are dimeric complexes with 2 CaMBDs and 2 CaMs with the N-lobe EF hands occupied by Ca^{2+} whereas in the absence of Ca^{2+} CaMBD/CaM are monomeric complexes (Fig.3B) (Schuhmacher et al., 2001).

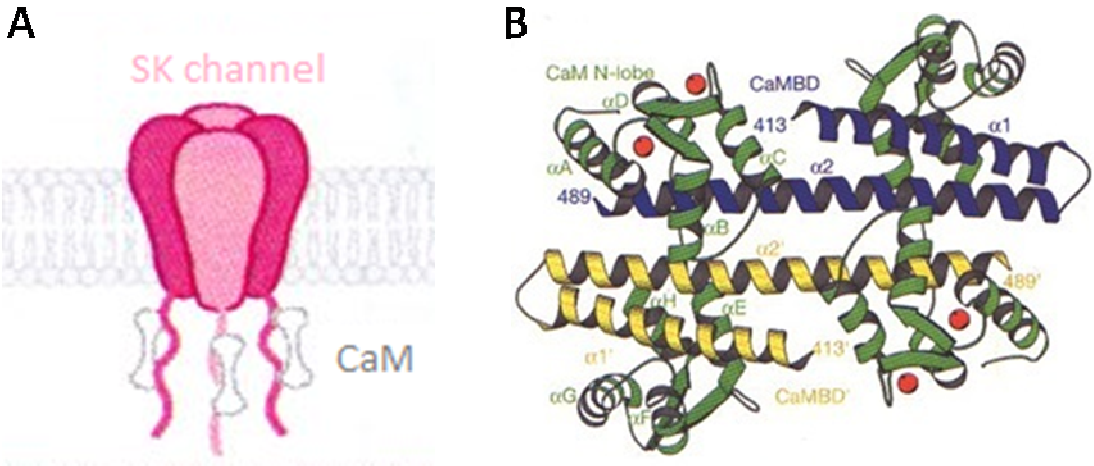


Fig. 3: Schematic representation of SK/CaM complexes. **A)** SK channels are composed of four pore-forming subunits, which have constitutively bound calmodulin (CaM) that mediates the Ca^{2+} gating (from Adelman et al., 2012, P248). **B)** Ribbon diagram showing the structure of the CaMBD/Ca^{2+}/CaM dimeric complex. In the complex, two CaMBDs are shown in blue and yellow, CaM molecules are green, and the Ca^{2+} ions are red. Secondary structural elements (α helices) of CaM and the CaMBD, and the first- and last-observed residues in the CaMBD, are labeled (from Schuhmacher et al., 2001, P1120).

Schuhmacher et al., (2001) proposed a chemo-mechanical model for the gating mechanism (Fig. 4), in which the binding of Ca^{2+} to each CaM N-lobe induces the exposure of its hydrophobic interfaces allowing it to interact with an adjacent CaMBD monomer forming

a dimeric complex. That dimerization creates a rotation that is transmitted to the corresponding S6 helices and opens the SK-channel gate (Schuhmacher et al., 2001).
Despite its role in the channel gating, the interaction of CaM is essential for the channels assembly and for their transport into the plasma membrane (Joiner et al., 2001; Lee et al., 2003).

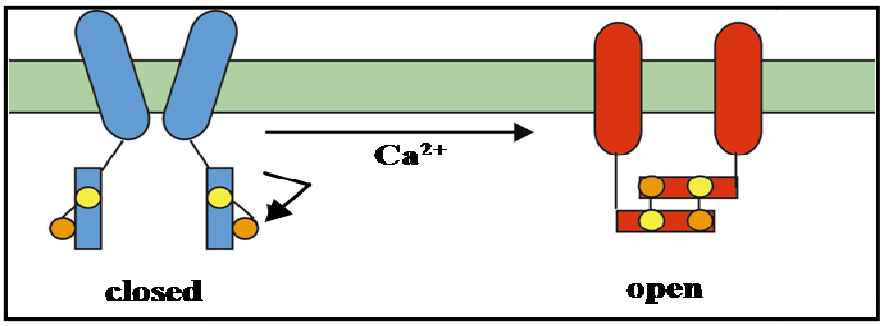


Fig. 4: Schematic representation of the proposed model of SK-channel gating (from Schumacher et al., 2001, P1123). The S6 regions that are linked to CaMBDs and the attached CaMs from two of the four SK-channel subunits are depicted. The C-lobe (yellow) and N-lobe (orange) of CaM are indicated by circles. Upon Ca^{2+} binding to the N-lobes of CaM, a dimeric complex is formed between two CaMBDs and Ca^{2+}–CaMs, inducing a rotation that is transmitted to the corresponding S6 helices and opens the SK-channel gate.

However, CaM is not the only protein binding to SK channels' C-termini. Proteomic analysis showed that, the cytoplasmic N and C termini of the functional SK channels bind constitutively to the protein kinase 2 (CK2) and protein phosphatase 2A (PP2A) which have opposing roles in regulation the Ca^{2+} gating (Bildl et al., 2004; Allen et al., 2007). When the channel is closed (Fig. 5), CK2 phosphorylates SK channel-associated CaM at threonine 80 (T80), reducing the apparent Ca^{2+} sensitivity and accelerating channel deactivation. In the open state, PP2A dephosphorylates CaM (T80) increasing thereby the Ca^{2+} sensitivity of the channel. This provides an example of strictly state-dependent modulation of ion channel gating and because the state of the channel is determined by the presence or absence of Ca^{2+} bound to CaM, the Ca^{2+}sensitivity of SK channels is itself Ca^{2+} sensitive (Allen et al., 2007; Adelman et al., 2012). Results from structure-function studies proposed that CK2 contacts three discrete points on the intracellular termini of the pore-forming subunits, whereas PP2A interacts with one discrete point, just distal to the CaMBD (Allen et al., 2007). Thus, these auxiliary components, CK2 and PP2A are

involved in the modulation of SK channels regulating therefore their activation. However, there is another way to modulate SK channels activity: SK channel-blockers and -activators. That is what I will discuss in the next section.

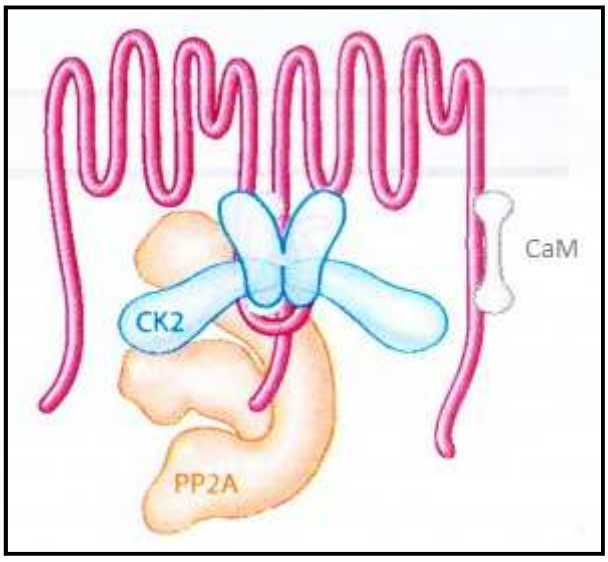


Fig. 5: Schematic model for the coassembled SK channel, CaM, CK2 and PP2A. The model represents two of the four subunits of an SK channels. CK2 and PP2A modulate the Ca^{2+} sensitivity of the channel by phosphorylating or dephosphorylating the SK-associated CaM (from Adelman et al., 2012, P248).

1.2.3 Pharmacology of SK channels

The first blocker of SK channels identified is apamin, an 18 amino acid peptide isolated from bee venom (Hugues et al., 1982). The three SK subtypes have different blocking sensitivity to apamin. Homomeric SK2 channels are the most sensitive, with an IC_{50} of 107 pM, whereas the IC_{50} for SK3 channels is 6.1 nM and ~10 nM for homomeric human SK1 channels (Lamy et al., 2010). Interestingly, both SK2 and SK3 have a much higher affinity for apamin binding (K_D ~ 5 pM) than blocking sensitivity (Lamy et al., 2010). The difference between the binding sensitivity and blocking affinity indicates that following apamin binding, blockade requires further interactions with the channel as explained below. Structure-function studies show that high affinity binding is mediated by three amino acid residues in the extracellular loop between transmembrane domains S3 and S4 (Nolting et al., 2007), but the actual block requires the subsequent interaction with residues in the outer pore of the adjacent subunit (Weatherall et al., 2011). This suggests that

apamin acts as an allosteric inhibitor, rather than a pore blocker as originally thought (Lamy et al., 2010; Weatherall et al., 2011). The importance of apamin is its high selectivity (Stocker et al., 1999), as it does not block any other type of ion channels (Yu et al., 2014) and the distinct values for IC_{50} and K_D suggest that apamin may be useful for determining the expression pattern of SK channel subtypes in native tissue. In addition, apamin has been used as therapeutic drug. It has been found that blocking of SK channels with apamin improves learning (Dechaux et al., 1997) and memory (Fournier et al., 2001).
In addition to blockade by apamin, SK channels are reversibly, but unselectively, blocked by tubocurarine, quaternary salts of bicuculline (Köhler et al., 1996; Khawaled et al., 1999; Seutin and Johnson 1999; Stroebaek et al., 2000), dequalinium and a large set of related bis-quinolinium cyclophanes (Dunn 1994; Campos Rosa et al., 2000). N-methyl-laudanosine, appears to be both selective for SK channels and reversible (Scuvée-Moreau et al., 2004). In addition, UCL 1848, a bis-quinolinium cyclophane, was found to block apamin-sensitive K^+ channels with nanomolar affinity (Chenet al., 2000). Another type of channel modulator acts by altering the Ca-sensitivity of the channel such as NS8593 which decreases the Ca^{2+}-sensitivity of SK channels (Stroebaek et al., 2000; Stroebaek et al., 2006).

On the other hand, different small organic compounds were found to enhance the Ca^{2+}-dependent activation of SK channels such as 1-ethyl-2-benzimidazolinone (1-EBIO) (Cao et al., 2001), the more potent 1-EBIO analogue, 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one (DC-EBIO) (Singh et al.,2001; Pedarzani et al., 2005), the centrally acting drugs zoxazolamine and chlorzoxazone (Cao et al., 2001), and 6,7-Dichloro-1Hindole-2,3-dione 3-oxime (NS309) (Stroebaek et al., 2004; Pedarzani et al., 2005). All these compounds enhance the SK channel activity by increasing their apparent Ca^{2+}-sensitivity (Cao et al., 2001).
Thus, considering the patho-mechanistic roles of the channels, modulators of SK channels may be developed as potential drug candidates for the treatment of many SK channel-related diseases, and may be used for investigating the expression pattern, structure, function, modulation, as well as for the explanation of the physiological role of SK channels.
As mentioned above, in my experiments I used SK3 channels. Therefore I will discuss these channels in the next sections.

1.3 SK3 channels

SK3 channel is characterized by its N-terminus, which is two times longer than those of SK1 and SK2 channels and contains two polyglutamine domains (CAG repeats). The first domain is not very polymorphic, its size varies between five to twelve repeats. While the second domain is highly variable with alleles containing 7 to 28 repeats (Chandy et al., 1998). These expanded polyglutamine tracts are detected in other proteins, which are related to neurodegenerative disorders such as Huntingtin disease, in which the N-terminus of the Huntingtin protein (Htt) contains an expanded polyglutamine repeats (The Huntington's Disease Collaborative Research Group, 1993). The sequence similarity between the N-terminus of SK3 channels and Htt is represented in Fig.6.

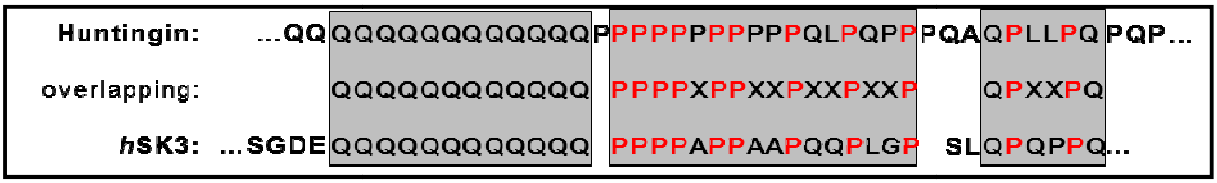


Fig. 6: Sequence comparison of Huntingtin and the N-terminal part of SK3 channels. The grey boxes indicate regions with high homology. Highlighted in red are homologous proline motives (from Janbein et al., 2014, P478).

In addition, Htt was found to interact with Endophilin A3 (Sittler et al., 1998), a protein of the Endophilin family (more details about Endophilin A3 will be discussed in the next section). That interaction is mediated by the SH3-domain of Endophilin A3 and the proline-rich region which directly follows the CAG-repeat in the N-terminus of Htt (Gao et al., 2006). Further studies 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 extended polyQ domain. Based on the CAG repeat- neurodegenerative disorders relation, it has been suggested that a prolonged CAG repeat in the N-terminus of SK3 channel is associated with schizophrenia or bipolar disorder (Chandy et al., 1998; Cardno et al., 1999). In contrast, other studies denied any influence on the developement of schizophrenia or bipolar disorder (Rohrmeier et al., 1999; Hawi et al., 1999; Glatt et al.,

2003). However, Grube et al (2011) showed that long CAG repeats in the N-terminal part of SK3 channels reduced SK3 channel function enhancing therefore the cognitive performance of schizophrenic patients, suggesting that SK3 genotypes might modify cognitive performance in schizophrenic patients.

SK3 channels are found in the laminas I, II and III of the spinal cord and in the periphirin-positive (c-type) and periphirin-negative (A-type) cells in the dorsal root ganglion. In these cells, active SK3 channels are found to control the sensory input into the spinal cord, as blocking SK3 channels by UCL 1848 increased sensory input and activating these channels by 1-EBIO reduced sensory input (Bahia et al., 2005). In addition, SK3 channels were expressed in dopaminergic neurons in a differentially pattern, i.e. SK3 levels in the substantia nigra were higher than in the ventral tegmental area (Wolfart et al., 2001). In dopaminergic neurons in the substantia nigra (DA SN), SK3 channels are responsible to control the frequency of spontaneous firing. In addition, SK3 channel activity was crucial to maintain the high precision of the intrinsic pacemaker of DA SN neurons (Wolfart et al., 2001).

The expression of SK3 channels is not restricted to excitable cells. Recent studies showed that SK3 channels are expressed at the luminal membrane of the distal tubule and the entire collecting duct system where BK channels are also expressed. Both channels are activated by TRPV4-mediated Ca^{2+} influx and function in a cooperative way to control the K^{+} excretion and the membrane voltage (Berrout et al., 2014).

In arteries, expression of SK3 channels is restricted to arterial endothelium where they contribute to the regulation of arterial tone and blood pressure (Taylor et al., 2003).
SK3 channels are also expressed in mouse myometrium where they have a pivotal role in regulating uterine contractility by limiting Ca^{2+} influx through L-type Ca^{2+}-channels and disrupting the development of concerted phasic contractile events (Brown et al., 2007). Interestingly, the expression levels and the density of SK3 channels in myometrium differ during pregnancy (Pierce et al., 2008). The authors determined that the expression level of SK3 channels must be down-regulated in the late phase of pregnancy to enable the uterus to generate forceful contractions required for parturition (Pierce et al., 2008). Thus, over-expression or the failure to down-regulate the expression of SK3 channels may delay the onset of labor or abrogate parturition by reducing the ability of the uterus to contract (Brown et al., 2007; Pierce et al., 2008).

So, these findings show that SK3 channels play important roles in several tissues, and that the expression levels and the density of these channels are essential factors for the channel's function. However, the mechanism controlling the level of expression and the density of SK3 channels is not determined yet.

1.4 Endophilins

Endophilins are a family of SH3-containing proteins. Three positive clones were identified SH3GL1, SH3GL2 and SH3GL3 or SH3p8, SH3p4 and SH3p13 corresponding to endophilins A2, A1 and A3, respectively (Micheva et al., 1997). Endophilin A2 (SH3GL1) is the most abundant isoform and is ubiquitously expressed in human tissues. However, the expression of endophilin A1 (SH3GL2) is restricted to the brain, while endophilin A3 (SH3GL3) is expressed in the brain and testis and may be in other tissues (Giachino et al., 1997, Ringstad et al., 1997).

Endophilins are composed of a BAR domain (Bin/Amphiphysin/Rvs) at the N-terminus part, variable region and a SH3 domain at the C-terminus part (Peter et al., 2004): the BAR domain mediates the binding to the lipid bilayer and is able to induce and stabilize membrane curvature and to bind to already curved membrane (Gallop et al., 2006; Masuda et al., 2006; Weissenhorn, 2005). Following the BAR domain there is a coiled-coil regions which mediate the dimerization of Endophilins, which may exist as homo- or heterodimers (Ringstad et al., 2001). . The variable region with an unknown higher-order structure is important in determining whether endophilin promotes or inhibits receptor-mediated endocytosis (Suguira et al., 2004). The SH3 domain mediates the interaction of endophilins with PRR (proline-rich region) of binding partner (Cicchetti et al., 1992)

Endophilins are considered as fundamental component of the clathrin-mediated endocytosis machinery (Verstreken et al., 2002), as they are involved in several stages of clathrin- mediated synaptic vesicle endocytosis, from generating membrane complexes to later events such as vesicle fission and uncoating (Ringstad et al., 1999). However, Endophilin A3 was found to act as negative regulator of clathrin-mediated endocytosis in brain neurons (Sugiura et al., 2004). Endophilins (BAR domain-containing proteins) are enriched at the presynaptic membrane, where they are important for the synaptic vesicle

endocytosis (Guichet et al., 2002). Interestingly, SK3 channels are also found at the presynaptic nerve terminal (Obermaier et al., 2003).

Since Huntingtin was shown to interact with Endophilin3 (Sittler et al., 1998), fellow worker in our laboratory provided first evidence about a possible interaction of Endophilin A3 with SK3 channels in vitro. The interaction of Endophilin A3 with the N-terminal part of SK3 channel was determined using the yeast-two hybrid system (Spindler et al., 2003), and confirmed by a pull down assay (Hoppner et al., 2004). Furthermore, Endophilin A3 and SK3 channels were found to interact in vivo in PC12 cells (Abo Quader, 2013). The author showed that the interaction of Endophilin A3 with SK3 channels reduced whole-cell SK3 current in PC12 cells. The reason or mechanism for such a reduction in whole-cell SK3 current was not investigated (Abo Quader, 2013). Recently, the co-expression of Endophilin A3 and SK3 channels in mammalian cells in a close spatial proximity has been determined which supports the determined interaction of both proteins (Janbein et al. 2014).

1.5 Aim of the study

The sequence similarity between Huntingtin and the N-terminus part of SK3 channels and the fact that Huntingtin proteins interact with Endophilin A3 (Sittler et al., 1998) led fellow workers in our laboratory (Grissmer´s laboratory) to investigate a possible interaction between SK3 channels and Endophilin A3. Thus, an interaction between the N-terminal part of SK3 channels and Endophilin A3 had been determined not only in vitro (Spindler et al., 2003; Hoppner et al., 2004) but also in mammalian PC12 cells (Abo Quader, 2013). Abo Quader (2013) found that the interaction of Endophilin A3 with SK3 channels reduced whole-cell SK3 current, however, did not investigate the reason or mechanism for such a reduction (Abo Quader, 2013).

Thus, based on these data, the aim of my work was to answer the following questions:

1. Is the membrane expression of SK3 channels changed by the interaction with Endophilin A3?
2. Is the Ca^{2+} sensitivity of SK3 channels modified by the interaction with Endophilin A3?
3. Does the interaction of Endophilin A3 modify the pharmacological characteristics of SK3 channels?

2 MATERIAL AND METHODS

2.1 Molecular Biology methods

2.1.1 Solutions and chemical components for molecular biology

All chemical components used in this study were purchased in high quality from the following companies: Merck KGaA (Darmstadt, Germany), Invitrogen Ltd. (Paisley, Scotland, UK), Carl Roth GmbH (Karlsruhe, Germany) and Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

Table 1: List of solutions and antibiotics used in the molecular biology experiments

Solutions	**Components**
Ampicillin stock solution	50 mg/ml sterile filtered solutions and stored at -20 °C.
Kanamycin stock solution	50 mg/ml sterile filtered solutions and stored at -20 °C.
LB medium, per liter	peptone (10 g), yeast extract (5 g), NaCl (5 g), H_2O (1 l), pH 7.2; autoclaved
LB agarsolution (LB agarplate), per liter	LB medium, Agar-Agar (20 g), pH 7.2; autoclaved
2YT medium	peptone (10 g), yeast extract (10 g), NaCl (5 g), H_2O (1 l), pH 7; autoclaved
EDTA stock solution 0.5 M	Ethylenediaminesodiumtetraacetate (93.05 g), H_2O (500 ml), pH 8
Ethidiumbromide	10 mg/ml
DNA ladder buffer (Loading buffer)	15 % Ficoll, 0.1 % Bromphenolblue
TBE buffer	Tris (89 mM), Boric acid (89 mM), EDTA (2 mM)
$CaCl_2$ solution	$CaCl_2$ (1.47 g), H_2O (100 ml); autoclaved

2.1.2 DNA clone

The plasmid vector used in my work was pTL1-HA-SH3GL3 coding for SH3GL3 and carrying an ampicillin resistance gene. That vector was a kind gift from Dr. Sittler from the Max-Planck-Institute for Molecular Genetics (Berlin, Germany). The PTL1-HA-SH3GL3 vector has an HA-Epitop on the 5'-end of SH3GL3. The PTL1 vector has a SV40 promotor for the in vivo protein expression, and the T7 Promotor for the in vitro expression. The pEGFP vector encoding for the green fluorescent protein and carrying a kanamycin resistance gene was used for visual selectivity of transfected cells.

2.1.3 Generation of competent cells and bacterial transformation

In order to prepare large quantities of any plasmid DNA, the plasmid DNA should be first introduced into competent bacterial cells as *E.coli* by a process called transformation. The transformation process requires LB medium, 2YT medium (Table 1), Agar-plates and competent cells.

A) Agar-plate preparation

To prepare the agar-plates, the LB-agar solution was first prepared (see Table 1). The solution was then autoclaved for 20 min at 120°C and 1.2 bar. After the solution was cooled to 55°C, the appropriate amount of the desired antibiotic was added. The addition of an antibiotic allows for the selection of only those bacteria which contained the plasmid carrying the antibiotic resistance gene. After mixing, the solution was poured into 90 mm sterile plates. The solidified plates were rested overnight at room temperature and then stored in plastic bags at 4°C.

B) Preparation of competent cells

Competent cells are bacterial cells chemically treated by calcium chloride that allow for foreign plasmid incorporation into the cells (Mandel and Higa, 1970). Bacterial cells used

in this work are *E.coli* cells (XL1-Blue strain). To prepare competent cells, 10 ml of LB medium (without antibiotic) were inoculated with one colony of the XL1-Blue strain and incubated at 37°C overnight at 200 rpm. Next day, 5 ml of the overnight culture was transferred into 500 ml LB medium and incubated at 37°C at 200 rpm until the optical density (OD) of the culture reached 0.3 to 0.5 (OD_{600}= 0.3 to 0.5). After an incubation on ice for 20 min, the culture was centrifuged at 4°C for 10 min at 3000 g. The supernatant was then poured off and pelleted cells were resuspended in 30 ml cold 0.1 M $CaCl_2$ and incubated again on ice for 30 min. The suspended cells were centrifuged again at 4°C for 10 min at 3000 g. After decanting the supernatant, cells were resuspended in 8 ml cold 0.1 M $CaCl_2$. Competent cells were stored as aliquots (150 µl/eppendorf tube) at -80°C.

C) Transformation of competent cells

The heat shock-transformation is one of different methods used to incorporate a plasmid DNA into a competent cell. For that process, frozen competent cells were thawed on ice. Then, 100 ng of the desired plasmid was added to 50 µl of thawed cells, gently mixed and incubated on ice for 30 min. A heat shock of the competent cells/plasmid DNA mixture for 45 s at 42°C, in a water bath, allows the plasmids to enter the bacterial cells. After an incubation on ice for 20 min, 500 µl of pre-warmed 2YT medium was added and the mixture was incubated on a shaker for 45 min at 37°C. That step ensures more transformation efficiency as it allows the bacteria time to generate the antibiotic resistance proteins encoded on the plasmid DNA so that they will be able to grow, once plated on the antibiotic containing agar-plates. After that step, 100 – 300 µl of the mixture was plated on agar-plates containing the appropriate antibiotic and incubated overnight at 37°C. On these plates, only the bacteria containing the plasmid DNA of interest will be able to grow and to build colonies.

2.1.4 Minipreparation of plasmid DNA

The plasmid DNAs used in that work are: the pEGFP encoding for the green fluorescent protein and carrying a Kanamycin resistance gene, and the pTL1-HA/SH3GL3 encoding for the Endophilin 3 protein and carrying an ampicillin resistance gene. These plasmid

DNAs were isolated using the GeneJET™ Plasmid Miniprep Kit (Fermentas GmbH, Germany).

Minipreparation of plasmid DNA with the GeneJET™ Plasmid Miniprep Kit is a quick DNA isolation method yielding micrograms of plasmid DNA. The purified DNA plasmid can then be used for transfection or other molecular cloning processes. For the plasmid DNA extraction, 5 ml of 2 YT medium containing the appropriate amount of the antibiotic were inoculated with one colony of bacterial cells transformed with the plasmid of interest and incubated at 37°C overnight at 200 rpm. On the next day, bacterial cells were pelleted by centrifugation at 13000 rpm for 15 min at 4°C. The isolation of the plasmid DNA with the GeneJET™ Plasmid Miniprep Kit (Fermentas GmbH, Germany) was performed according to the manufacture instructions. The concentration of the extracted DNA was determined by a photometry system (GeneQuant 1300 Photometer, GE Healthcare, München, Germany).

2.2 Cell Culture

2.2.1 Solutions and chemical components

For culturing and preparing PC12 cells for patch clamp experiments, the following culture medium and chemical components were used:

Table 2: List of culture medium and chemical components used in the cell culture experiments

Minimum Essential Medium [+] Earle's Salts (MEM + GlutaMaxTM)	Gibco by life technologies, UK
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific, Bonn, Germany
Horse Serum (HS)	Gibco by life technologies, UK
Phosphate Buffered Saline without calcium (PBS)	PAA Laboratories GmbH, Pasching, Austria
poly-L-lysine	Sigma-Aldrich Chemie GmbH, Steinheim, Germany

Complete culture medium is the MEM medium supplemented with 10 % FBS and 5 % HS. The complete culture medium was used to culture and maintain the PC12 cells. To improve the cell adhesion for the patch clamp experiments, glass chamber were covered with poly-L-lysine.

2.2.2 Culture of PC12 cells

The PC12 cell line is derived from a pheochromocytoma of the rat adrenal medulla. PC12 cells were purchased from the company ATCC (LGC standards GmbH, Wesel, Germany) and stored in liquid nitrogen. For culturing, cells were removed from the liquid nitrogen and rapidly thawed in a 37°C water-bath. Once thawed, cells were transferred into a 15 ml conical tube containing 9 ml of Complete Culture Medium and centrifuged for 5 min at 13000 rpm. The supernatant was removed and the cell pellet was re-suspended in 10 ml of complete culture medium. Cells were then transferred into a 50 ml culture flask and incubated at 37°C in an incubator with a CO_2 partial pressure of 5 %. Once cells are about 80 % confluent, they were able to be used. For cell splitting, the old medium was aspirated; cells were detached by gentle taps of the culture flask and then re-suspended in 10 ml of complete culture medium. After re-suspension, cells were transferred into new 50 ml flasks in a 1:10 dilution and into several 35 mm dishes for transfection.

2.2.3 Transfection

For patch clamp experiments, PC12 cells were transfected with ~ 0.5 µg pEGFP alone (as control cells) or with 0.5 µg pEGFP and 1 µg pTL1-HA/SH3GL3 using lipofectamine as transfection reagent. For transfection of each dish, 95 µl of the MEM-medium were mixed with 5 µl Lipofectamine in an Eppendorf tube and incubated at room temperature (RT) for 5 min. Then, the desired plasmid DNA was added and incubated again at RT for 20 – 30 min. Before adding the mixture to cells prepared in a 35 mm dish, the old medium was replaced by a new complete culture medium.

2.2.4 Preparation of cells for the electrophysiological measurement

For the electrophysiological measurements, transfected cells were plated onto a glass chamber. Glass chambers were prepared by glueing a Teflon ring (O-ring PTFE N. DIN, THF GmbH & Co. KG, Ulm, Germany) onto a glass cover slip (24x24 mm, thickness 0.13-0.16 mm, VWR International GmbH, Darmstadt, Germany) using a silicon elastomer (Sylgard 186, Dow Corning, Inc., Midland, USA). For better adhesion of cells, the glass chamber was coated with poly-L-lysine as follows: ~ 400 µl poly-L-lysine was added onto the glass chamber, incubated at RT for 30 min. Afterwards, poly-lysine was removed; the glass chamber was washed with sterile water and then left under the laminas flow hood to dry. To transfer the cells, the medium was removed from the culture dish, cells were detached by gentle taps and re-suspended in 5 ml complete culture medium. From this suspension, 100 - 200 µl were transferred onto a coated glass chamber and mixed carefully with ~300 µl culture medium.

2.3 Electrophysiology

2.3.1 Solutions and chemical components for electrophysiology

All patch-clamp measurements were carried out at room temperature (20 – 25°C). SK3 currents were activated using an internal pipette solution containing (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. SK3 currents were measured in the following external solution: The Na^+ aspartate solution (Na-Asp) contained (mM): 160 Na^+ aspartate, 4.5 K^+ aspartate, 2 $CaCl_2$, 1 $MgCl_2$ and 5 HEPES, pH 7.4. 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.

For the Ca^{2+} measurements, first the tip of the pipette was filled with 2 to 4 µl of a solution containing (50 µM fura-2, 135 mM potassium aspartate, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, pH 7.2), then tip filling the pipette with another solution containing (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). Fura-2 was obtained from Molecular Probes (Eugene, OR).

2.3.2 Patch-clamp set-up

The electrophysiological measuring system consisted of an inverted microscope, the Axiovert 100 (Carl Zeiss GmbH, Oberkochen, Germany). The microscope was attached to an anti-vibration table (Newport Corporation, Irvine, USA). Mounted to the stage of this microscope was a micromanipulator (Märzhäuser GmbH & Co. KG, Wetzlar, Germany). The microscope was equipped also with an epifluorescent illumination to allow the visualization of GFP-cotransfected cells. Membrane currents were measured by an EPC-9 patch clamp amplifier (HEKA Electronik, Lambrecht, Germany) interfaced to a PC running acquisition and analysis software (Patchmaster / Fitmaster v2.00). All metal parts near the head-stage were grounded in order to reduce the disruptive noise. The micropipettes were pulled (in three steps) from borsilicate glass capillaries (GB150-TF10, Science Products GmbH, Hofheim, Germany) using a DMZ Univeral Puller (Zeitz-Instruments GmbH, Munich, Germany). The micropipettes were fire polished under optical examination on a self-made microforge. The resistances of the microelectrodes were in the range of 2 - 4 megaohms (MΩ). The pipette electrode consisted of a chlorinated silver wire surrounded by pipette solution. The bath electrode was prepared from a 2 - 3 cm long chlorinated silver wire joined to a standard 2 mm jack. This chlorinated silver wire was placed into a PVC-tube and filled with hot bath solution (Na-Ringer) containing 1 % agarose. The bath electrode was regularly replaced to avoid electrical offsets and offset drifts. All currents were filtered by a 2.9-kHz Bessel filter and recorded at a rate of 1-5 kHz. Patch-clamp experiments were done with the whole cell recording mode of the patch-clamp technique (Hamill et al., 1981) at room temperature. The bath solution was exchanged using a simple syringe–driven perfusion system.

The data analysis was performed with the program Fitmaster v2.00 (HEKA Elektronik GmbH, Lambrecht, Germany), the software Igor Pro 3.12. (WaveMetrics Inc., Lake Oswego, Oregon, USA), and Microsoft ® Office Excel 2007 (Microsoft Corporation, Seattle, USA).

All values presented in that work are mean ± SD with “n” the number of independent observations. K_D values were deduced by fitting a modified Hill equation, $I_{rel} = 1/(1 +$

(K_D/ [DC-EBIO])). $I_{rel} = (I-I_{min})/(I_{max}-I_{min})$ with I_{min} the current obtained at -80 mV without DC-EBIO and I_{max} the current obtained at -80 mV with 1000 µM DC-EBIO.

2.4 Calcium imaging

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined as described before (Frei et al., 2006). 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 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} 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). The values for K_{eff}, R_{min}, and R_{max} were calculated to be 1.134 µM, 0.1, and 1.74.Curves were fitted according to the equation $g/g_{max} = A/(1+(EC_{50}/[Ca^{2+}])^{nH})$, where g_{max} is the maximal conductance, A is determined by the fit of the curve, EC_{50} is the half-maximal activating Ca^{2+} concentration and nH is the Hill coefficient.

3 RESULTS

3.1 Does Endophilin A3 reduce the SK3 channel activity through endocytosis?

Endophilin A3 is thought to be involved in the endocytosis. So, its interaction with SK3 channels might lead to the endocytosis of the channels, which will result in a decreased SK3-current. To determine that, I used PC12 cells which express endogenous SK3 channels. As a negative control, to exclude a non-specific effect of the vector on the expression of endogenous SK3 channel, I transfected PC12 cells just with the GFP protein-expressing vector pEGFP-N1. To determine the effect of Endophilin A3, I transfected PC12 cells with both the Endophilin A3-encoding vector pTL1-HA/SH3GL3 and pEGFP-N1 (for visual identification of transfected cells under a fluorescent microscope). PC12 cells contain endogenous KIR channels. Since Cs^+ was described to block KIR channels (McCloskey and Cahalan, 1990), and SK3 can carry a significant Cs^+ current (Wittekindt et al., 2004a), SK3 conductance was measured in Cs^+ aspartate solution. The currents are recorded with a 200 ms voltage ramp from -120 mV to +60 mV. The main thing in these experiments is to compare the inward SK3 currents therefore I will show only the voltage range between -120 to 0 mV (Fig7B).

Fig. 7 shows the representative whole-cell currents in Na^+ solution and in Cs^+ solution in control transfected PC12 cells. SK3 channels were activated by pipette solutions containing 1 μM free Ca^{2+}. After break-in to the whole-cell mode, the current was first measured in Na^+ solution (- 19 pA at -80 mV). When the extracellular solution is exchanged for a Cs^+ solution, the SK3-specific inward current increased monotonically with hyperpolarized values (-250 pA at -80 mV) (Fig. 7). To calculate the mean of the SK3 current amplitudes I measured the currents in Cs^+ solution in 18 control transfected PC12 cells. The mean of SK3 current amplitudes at -80 mV was -350 ± 150 pA (mean ± SD, n=18).

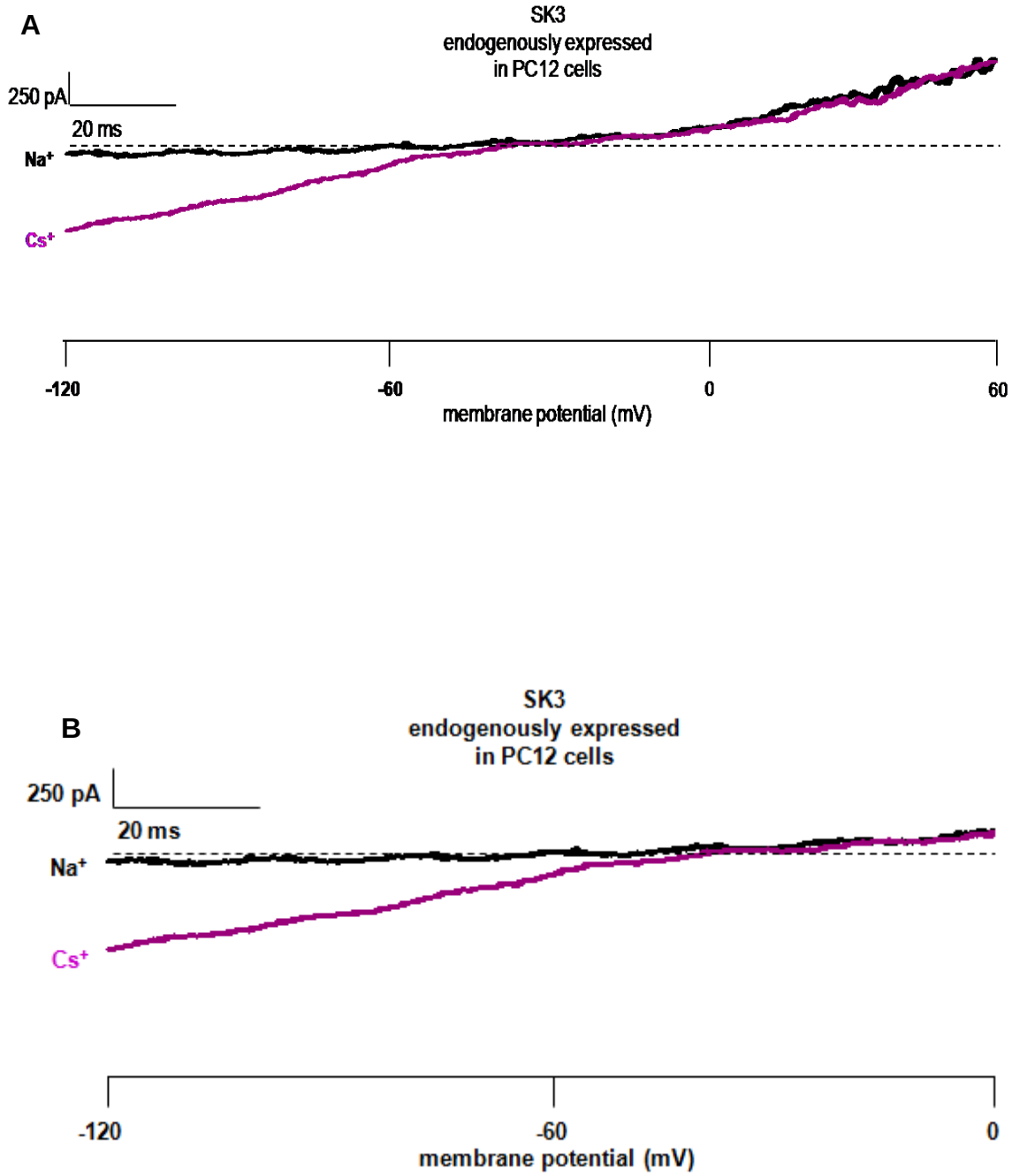


Fig. 7: Representative currents through endogenously expressed SK3 channels in control transfected PC12 cells. SK3 channels were activated by pipette solutions containing 1 µM free Ca^{2+}. Whole-cell currents were elicited with 200 ms voltage ramps from -120 to +60 mV (A) in the presence of 160 mM extracellular Na^+-solution (Na^+) and 160 mM extracellular Cs^+-solution (Cs^+). For better visualization only the voltage range between -120 to 0 mV is shown (B).

To determine the effect of Endophilin A3, I transfected PC12 cells with both vectorspTL1-HA/SH3GL3 and pEGFP-N1 (for visual identification of transfected cells under a fluorescent microscope) vector.PC12 cells transfected with Endophilin A3 showed in Na^+ solution at -80 mV a current-amplitude of -10 pA (Fig. 8). In contrast to the current obtained in control cells, the SK3-specific inward current did hardly increase in Cs^+ solution (Fig. 8), indicating little activation of current through SK3 channels. These results confirm the results obtained previously by Abo Quader (2013). The number of measured cells transfected with Endophilin A3 was 24 cells. The mean of SK3-specific Cs^+-current amplitudes at -80 mV was-80 pA ± 60 pA.

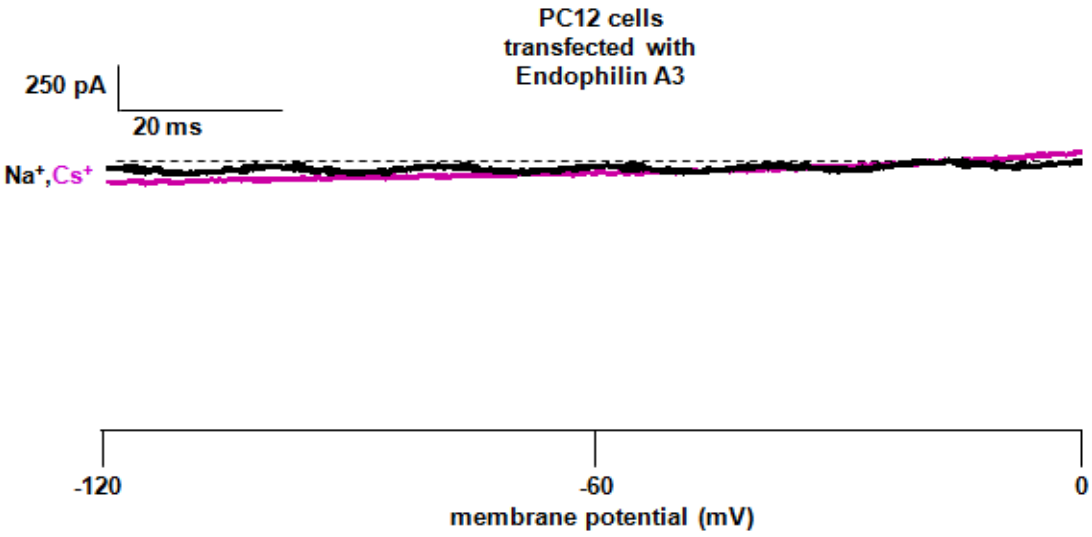


Fig. 8: Representative currents through endogenously expressed SK3 channels in PC12 cells transfected with Endophilin A3. SK3 channels were activated by pipette solutions containing 1 µM free Ca^{2+}. Whole-cell currents were elicited with 200 ms voltage ramps from -120 to +60 mV in the presence of 160 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.

The results shown in Fig 8 might indicate that Endophilin A3 removed SK3 channels from the membrane. In that case, the application of SK3 channel activator (i.e DC-EBIO) should not increase or should hardly increase the SK3-specific current (considering that some SK3 channels might still be in the membrane). However, the application of a Cs^+ solution containing 30 µM DC-EBIO resulted in an increase in SK3-specific current to a value close to that obtained in control transfected cells with Cs^+ solution alone (Fig. 9).

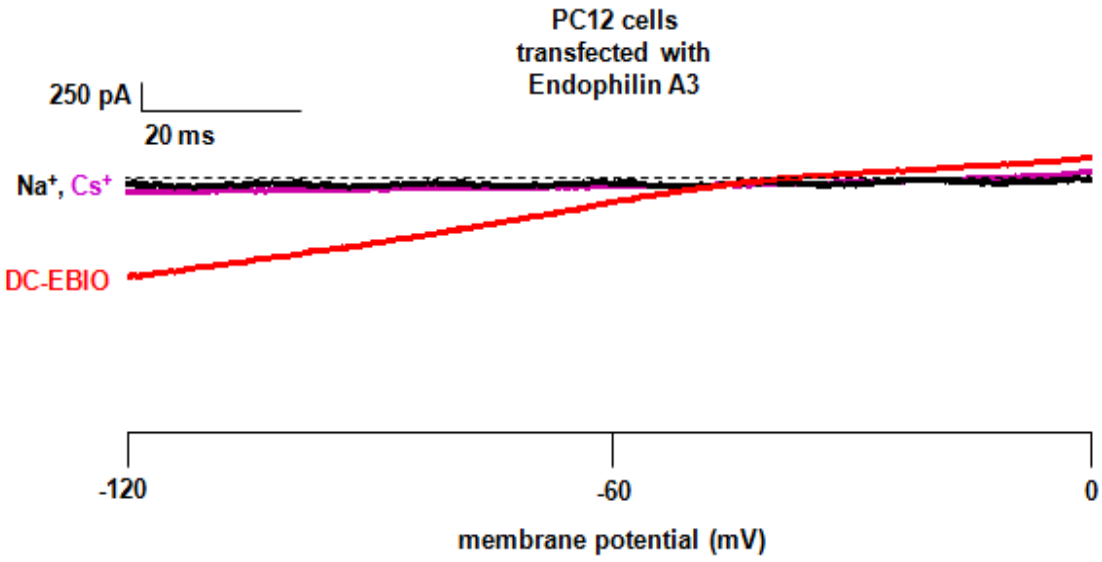


Fig. 9: Representative currents through endogenously expressed SK3 channels in PC12 cells transfected with Endophilin A3. SK3 channels were activated by pipette solutions containing 1 µM free Ca^{2+}. Whole-cell currents were elicited with 200 ms voltage ramps from -120 to +60 mV in the presence of 160 mM extracellular Na^+-solution (Na^+) and 160 mM extracellular Cs^+-solution (Cs^+) and in Cs^+-solution containing 30 µM DC-EBIO. For better visualization only the voltage range between -120 to 0 mV is shown.

The results shown in Fig. 9 disprove the hypothesis that the SK3 channels might be removed from the plasma membrane by Endophilin A3. As the Cs^+ solution containing 30 µM DC-EBIO was applied to the same cells used for Fig. 8 after reaching the steady state in Cs^+-solution, the number of measured cells was also 24. The mean of SK3 current amplitudes at -80 mV was-360 pA and the standard deviation was ± 270 pA.

For a better comparison of the SK3 currents, I collected the three figures (7, 8 and 9) in one figure (Fig.10).

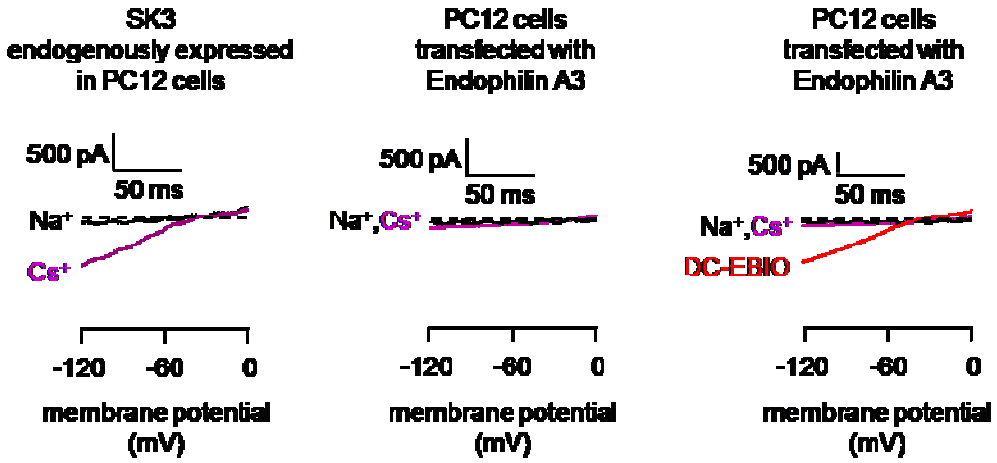


Fig. 10: Representative currents through endogenously expressed SK3 channels in control transfected PC12 cells (left panel), in Endophilin A3 transfected PC12 cells (middle panel), and in Endophilin A3 transfected PC12 cells in the presence of 30 µM DC-EBIO (right panel). SK3 channels were activated 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^+) and in Cs^+-solution containing 30 µM DC-EBIO (right panel). For better visualization only the voltage range between -120 to 0 mV is shown (from Janbein et al., 2014, P481).

For a better comparison, I summarized the data obtained from all experiments as a scatter plot (Fig.11). SK3 current amplitudes of control transfected PC12 cells at -80 mV were -350 ± 150 pA (n=18), whereas current amplitudes in PC12 cells transfected with Endophilin A3 were found to be a lot smaller (-80 ± 60 pA, n=24) compared to control transfected PC12 cells.

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). These results indicate that Endophilin A3 reduced the activity of endogenous SK3 channels functional in the membrane of PC12 cells under our measuring conditions.

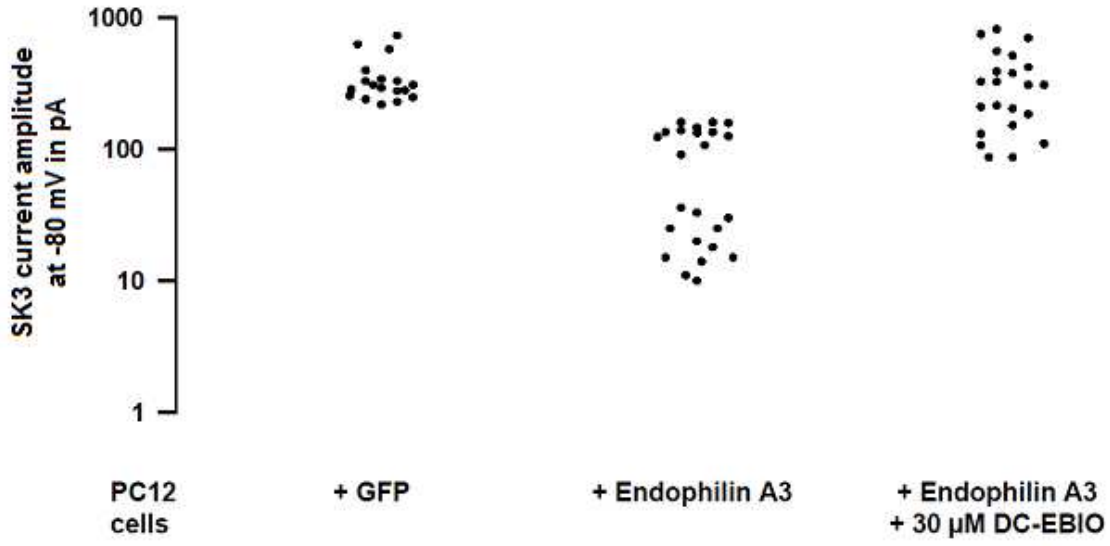


Fig. 11: Scatter plot of specific SK3 current amplitudes in Cs^+ solution at -80 mV in PC12 cells from the experiments shown in Fig. 7, 8, and 9 and similar experiments (from Janbein et al., 2014, P481). Control transfected (GFP) PC12 cells (left); Endophilin A3 transfected PC12 cells without (middle) and with 30 µM DC-EBIO (right).

Since Endophilin A3 did not remove SK3 channels from the membrane, how did it act to reduce the activity of functional SK3 channels in PC12 cells transfected with Endophilin A3?

3.2 Does the interaction of Endophilin A3 with SK3 channels change the Ca^{2+} sensitivity of SK3 channels?

In order to find out, if the Ca^{2+} sensitivity of the SK3 channels is changed in the presence of Endophilin A3,I measured simultaneously the whole-cell SK3 conductance (Fig. 12, A and B) and the internal Ca^{2+} concentration in the same cell of control transfected as well as of Endophilin A3 transfected PC12 cells. The benefit of this procedure is that the current amplitude can be rapidly correlated 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 F350 and F380 of the fura-2 measurements were used 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 nH is the Hill coefficient (Fig. 12, C and D). A was determined by the fit and was 1.0 for control transfected PC12 cells (Fig. 12C) and 1.3 for Endophilin A3 transfected PC12 cells (Fig. 12D). Endophilin A3 expressed cells show a higher EC_{50} and Hill coefficient than control transfected cells (EC_{50} = 2.3 ± 0.04 μM, Hill coefficient = 5.6 ± 1.5; EC_{50} = 1 ± 0.1 μM, Hill coefficient = 4.6 ± 0.2, respectively). Since, EC_{50} reflects the Ca^{2+} sensitivity of SK3 channels, I conclude from these results that the interaction of Endophilin A3 with SK3 channels made the channels less sensitive to Ca^{2+}. Since the Ca^{2+}-sensitivity of SK3 channels depends on the Ca^{2+}/Calmodulin binding site located at the C-terminus of the SK3 channels (Schumacher et al., 2001; Maylie et al., 2004; Adelmann et al., 2012) it might be possible that other properties of SK3 channels linked to that region of the channel like the activation by DC-EBIO (Pedarzani et al., 2001) might also be modified.

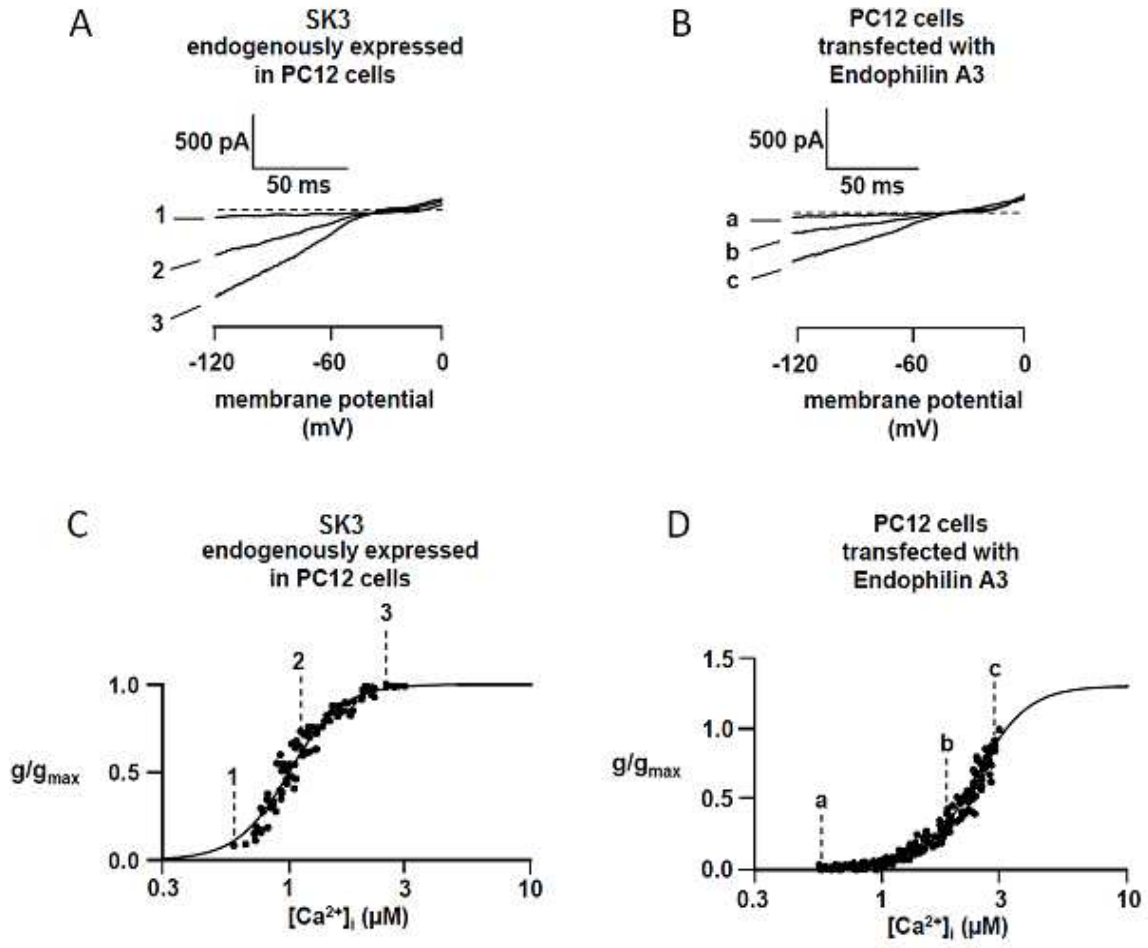


Fig. 12: Activation of SK3 channels in PC12 cells control transfected (GFP) or transfected with Endophilin A3 by internal Ca^{2+} (from Janbein et al 2014, P482). Membrane potentials were ramped from -120 to +60 mV in 200 ms. In parallel, the internal Ca^{2+} concentration was measured by the fura-2 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 SK3 current in control transfected cells. (B), Representative endogenous SK3 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 nH is the Hill coefficient. 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.

3.3 Is the DC-EBIO sensitivity of SK3 channels modified by the interaction with Endophilin A3?

The results showed in the previous Fig. (Fig. 12) demonstrate that Endophilin A3 changes the Ca^{2+} sensitivity of SK3 channels, which is conferred by the Ca^{2+}/Calmodulin binding site located at the C-terminus of SK3 channels (Schumacher et al., 2001; Maylie et al., 2004; Adelmann et al., 2012), I assumed that the DC-EBIO sensitivity might also be changed by the interaction of Endophilin A3 since this sensitivity has also been linked to the same region of the channel (Pedarzani et al., 2001). Therefore, I expressed Endophilin A3 in PC12 cells and investigated if there is an interference of Endophilin A3 with the DC-EBIO binding to SK3 channels. SK3 channels were activated by pipette solution containing 1 μM free Ca^{2+}. SK3 currents were first elicited in Na^+ solution, then in Cs^+ aspartate solution. After reaching the steady state, 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, I 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. 13A, shows a representative ramp current trace through endogenous SK3 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. 13B). 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). In similar experiments I obtained in control transfected cells 770 ± 295 pA (n=18) and in Endophilin A3 transfected cells 640 ± 485 pA (n=25) indicating hardly any loss of functional channels in the membrane by Endophilin A3. Therefore, I cannot exclude a minor loss of functional channels of the membrane of Endophilin A3 transfected cells.

To determine if there is any change in the DC-EBIO sensitivity of SK3 channels, I generated the concentration-response curves (Fig. 13 C and D). These concentration-

response curves were generated from the current records shown in Fig. 13 A and B and similar records. The calculated relative currents (I_{rel} = (I-I_{min})/(I_{max}-I_{min})) were plotted against the DC-EBIO concentrations (Fig. 13, C and D). 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), I conclude that the DC-EBIO sensitivity of SK3 channels did not change through the interaction with Endophilin A3.

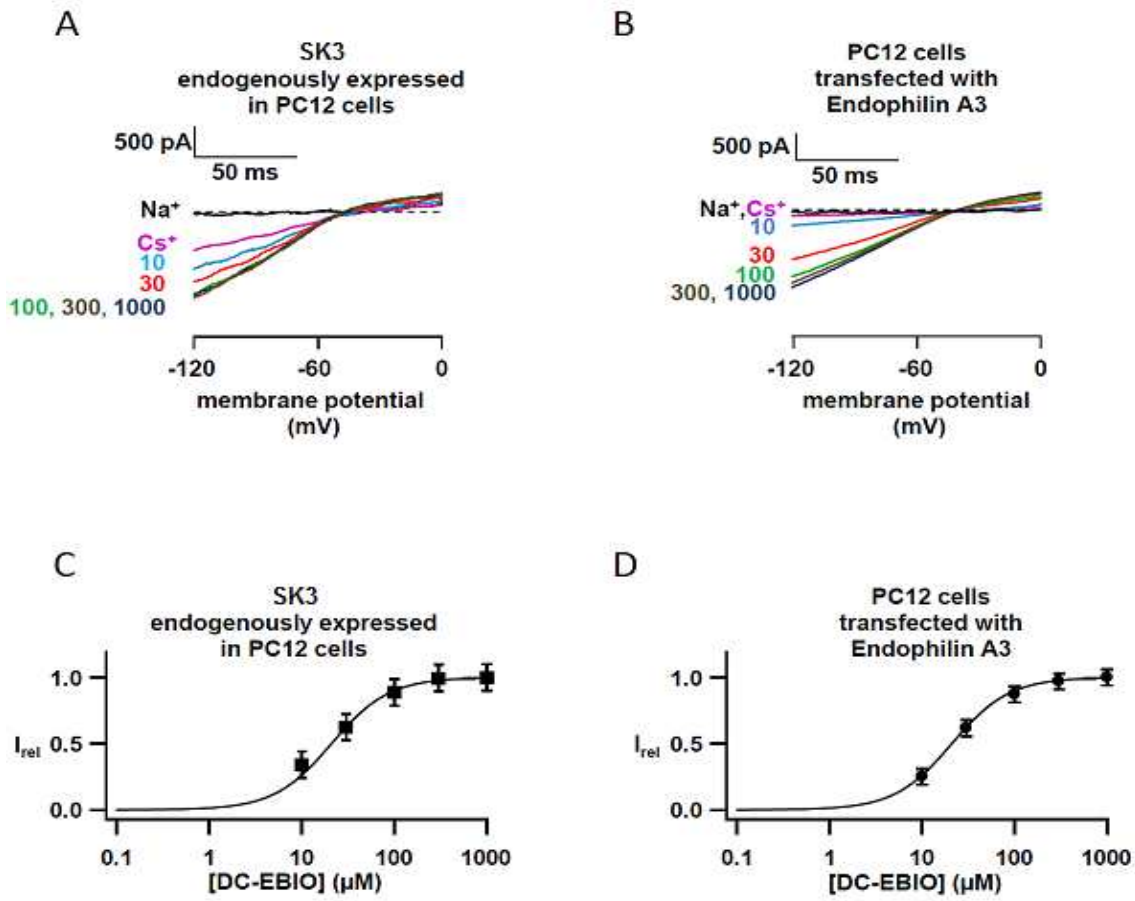


Fig. 13: Activation of endogenous SK3 currents in PC12 cells with different DC-EBIO concentrations (from Janbein et al., 2014, P483). Representative endogenous SK3 currents in control (GFP) transfected PC12 cells (A) and in PC12 cells transfected with Endophilin A3 (B). SK3 channels were activated by pipette solutions containing 1µM free Ca^{2+}. SK3 currents were elicited in Na^+ solution, in Cs^+ solution, then in Cs^+ solutions containing different DC-EBIO concentrations. (C,D) Concentration-response curves for the activation of SK3 current by different DC-EBIO concentrations (in comparison to the activation of SK3 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-I_{min})/(I_{max}-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 / [DC-EBIO])). The dissociation constant K_D for DC-EBIO was: 19 µM for control transfected PC12 cells (C), and 21 µM for Endophilin A3 transfected cells (D).

4 DISCUSSION

4.1 Impact of the interaction of Endophilin A3 on the function of SK3 channels

In this work, I could confirm the interaction of Endophilin A3 with SK3 channels which is determined previously by fellow worker in our laboratory. Therefore, I wanted to know the physiological meaning of that interaction. Endophilin A3 belongs to the family of endophilin proteins (Giachino et al., 1997). To that family belong also Endophilin A1 and A2, which are essential components of the endocytotic machinery (Ringstad et al., 1997; Verstreken et al., 2002; Schuske et al., 2003). Accordingly, I supposed that, like Endophilin A1 and A2, Endophilin A3 might induce endocytosis of SK3 channels. However, my patch-clamp experiments invalidated that hypothesis, since the SK3 current recorded in Endophilin A3 transfected PC12 cells increased after the application DC-EBIO to values close to those obtained in control transfected cells in Cs^+ solution alone. In addition, my experiments showed that Endophilin A3 changed the Ca^{2+} sensitivity of SK3 channels without affecting their DC-EBIO sensitivity. These results indicate that Endophilin A3 did not remove the SK3 channels from the membrane by endocytosis, which seems to be in agreement with studies indicating that Endophilin A3 inhibits the endocytosis (Sugiura et al., 2004), rather it reduced the activity of functional SK3 channels in the membrane of PC12 cells by changing their Ca^{2+} sensitivity. So, how did Endophilin A3 change the Ca^{2+} sensitivity of the SK3 channels? The best explanation of the impact of the interaction of Endophilin A3 on the Ca^{2+} sensitivity of SK3 channels is as described by Janbein et al., (2014). Authors supposed the following scenario: They assumed that the binding of Endophilin A3 to the N-terminus of SK3 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. CaM constitutively interacts with K_{Ca} channels and serves as Ca^{2+} sensor. A tetrameric SK3 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 hSK2 R419-L440, (hSK3 R572-L593) and with its N-terminal domain (N-lobe) more distal around hSK2 E469-Q487, (hSK3 E622-Q640). The N- and C-lobe of CaM are connected by a-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 (Zhang et al., 2012). New protein crystal structures identified a region in SK2 channels between S6 and the CaMBD important for the coupling in Ca^{2+} sensing by CaM and the mechanical opening of K_{Ca} channels (Zhang et al., 2013). In addition, this region is also involved in the binding of the DC-EBIO-related compound NS309 (Zhang et al., 2013). The authors could show that an intrinsically disordered fragment near S6, the so-called IDF region (hSK2 A403-M412 equivalent to hSK3 A556-M565) adapts a unique ordered conformation upon NS309 binding and that a C-terminal region of the SK2 channel close to the membrane (E399-K402 equivalent to E552-K555 in hSK3) interacts like a cuff with the CaM linker region after the proper CaM-CaMBD complex has been formed upon Ca^{2+} binding to CaM (Zhang et al., 2013). This interaction between the cuff and the CaM linker seems to contribute directly to the mechanical opening of SK2 channels and can also explain that phosyphorylation of CaM by CK2 reduces Ca^{2+}-sensitivity of SK2 channels by interfering with the mechanical coupling process rather than with Ca^{2+} binding to CaM (Bildl et al., 2004; Allen et al., 2007). This scenario might also explain my results, where a change in Ca^{2+}-sensitivity of SK3 by Endophilin A3 was observed, which could be overcome by DC-EBIO. In this case I 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.

4.2 Q14 vs Q19

As mentioned in the introduction part, the N-terminal region of the SK3 channel contains two polyglutamine-repeats, linked by PXXP motives. The second polyglutamine repeat is highly polymorphic. In the population, repeat lengths from 12 to 28 were identified, the modal repeat length is coding for 19 Gln (Chandy et al., 1998) or 18 Gln (Grube et al., 2011). Frei et al., (2006) showed that, an $SK3N_{(1\text{-}299),\ Q19}$ construct interacts with Endophilin A3 whereas $SK3N_{(1\text{-}274),\ Q14}$ did not. The authors suggested that, 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) (Frei et al., 2006). They also suggested that an

influence of the glutamine stretch would imply that individuals with shorter glutamine stretches would experience a higher SK3 activity that might influence their cognitive performance especially in schizophrenia as has been suggested (Grube et al., 2011).

4.3 Physiological roles of the interaction of Endophilin A3 with SK3 channels

Based on the results obtained from my experiments and that SK3 channels are colocalized with Endophilin A3 in nerve terminals of mouse hippocampal neurons (Roncarati et al., 2001; Obermaier et al., 2003), I could assume that the functional blockade of SK3 channels by Endophilin A3 may enhance signal transmission in neurons where SK3 channels control synaptic plasticity as has been shown in mice, where the blockade of SK3 channels with apamin, a specific SK channels blocker, facilitated memory encoding and learning (Messier et al., 1991; Deschaux et al., 1997), and increased dopamine release (Stocker et al., 1999;Pedarzani et al., 2001). In addition, the activation of SK3 channels with 1-EBIO or CyPPA impaired the encoding of object memory (Kyle et al., 2010). Therefore, the findings of Endophilin A3 reducing SK3 channel function might be an important modulator of signal transduction in neurons. In addition, the interaction might have an influence on a variety of cell function as described below.

4.3.1 Filopodia formation

In neural stem cells, activation of SK3 channels accelerated the local outgrowth of long filopodial processes (Liebau et al., 2011). In these cells, it has been found that SK3 channels interact with nWASP and Abi-1 proteins to build a complex which regulates filopodia formation. The Abi-1 protein interacts via its SH3 domain with the prolin rich N-terminal of SK3 channels. It is easy to imagine that either the functional down-regulation of SK3 activity via Endophilin A3 or the interaction itself by preventing the Abi-1 protein interaction could modulate or even prevent filopodia formation. The nWASP/Abi-1/SK3 complex was also found in developing hippocampal neurons where it regulated the neurite outgrowth through regulating the local rearrangement of the

cytoskeleton in early steps of neuronal differentiation. Therefore the interaction of SK3 with Endophilin A3 might also have an impact on differentiation.

4.3.2 Podosome formation

Siddiqui and collegues (2012) demonstrated a variety of molecules associated with podosome formation in microglia. The newly found core components seemed to be related to Ca^{2+} signaling (Orai1, STIM1, Calmodulin, Iba1) and also included the SK3 channel. Since SK3 channel inhibition did reduce invasion of the microglia it seems likely that an interaction of Endophilin A3 with SK3 that leads to a functional inhibition of SK3 channels would inhibit the migration of microglia (Siddiqui et al., 2012).

4.3.3 Cancer cell migration

Similar to the podosome formation described above (Siddiqui et al., 2012) an SK3-Orai1 complex seem to be important in cancer cell migration (Chantome et al., 2013). Earlier reports had established SK3 channels to control cancer cell migration (Potier et al., 2006; Girault et al., 2012) and the recent results suggested that SK3 channels control this cancer cell migration by controlling Ca^{2+} entry through an interaction with the OraiI channel within lipid rafts (Chantome et al., 2013). A lipid-raft disrupting agent, Ohmline, moved the SK3-Orai1 complex away from lipid rafts, and SK3-dependent Ca^{2+} entry, migration, and bone metastases were subsequently impaired (Chantome et al., 2013). It seems obvious that the interaction of Endophilin A3 with SK3 channels could influence the function of such a complex of ion channels thereby modulating migration and ultimately modulate the development of metastases.

4.3.4 Lipid rafts/Caveolae

Lipid rafts and Caveolae in Ca^{2+} signaling microdomains have already been discussed and their importance in organizing and regulating ion channels has recently been highlighted

(Pani et al., 2009). For SK3 channels it was shown that these channels are localized to Caveolae (Absi et al., 2007; Balut et al., 2012; Gao et al., 2012) and that these channels undergo caveolae-dependent endocytosis and not clathrin-mediated endocytosis (Gao et al., 2012) as one way to modulate the number of functional channels at the plasma membrane thereby influencing cell behavior including migration. In that context it might reasonable to envision that Endophilin A3 by interacting with SK3 channels will modify the normal trafficking of SK3 channels via Caveolae.

4.4 Physiological roles of Endophilin A3

4.4.1 Lamellipodia protrusion

Similar to the filopodia formation, lamellipodia protrusion is controlled by lamellipodin which was recently shown to interact with Endophilin A3 (Vehlow et al., 2013). The authors apparently identified a pathway in which Lamellipodin functions downstream of endophilin to regulate the F-actin cytoskeleton to support clathrin-mediated endocytosis during endocytosis of the epidermal growth factor receptor (EGFR). In such a setting, the interaction of Endophilin A3 with SK3 channels could also influence endocytosis thereby affecting development as well as cancer.

4.4.2 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 (2003) 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 (Chen et al., 2003). 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 (Tian et al., 2012). It is, however, unclear whether this interaction between Endophilin A3 and the Ca^{2+} channel has any influence on channel activity.

5 CONCLUSION

Endophilin A3 belongs to a family of BAR- and SH3 domain containing proteins. To that same family belong also Endophilin A1 and A2 which are involved in the process of vesicle scission in clathrin-mediated endocytosis. However the exact role of Endophilin A3 is not known yet. Endophilin A3 was found to interact with the Huntingtin protein (Sittler et al., 1998) and interestingly, the N-terminus of the SK3 channel shows high homology to Huntingtin. Therefore an interaction between Endophilin A3 and SK3 channels seemed possible. Such an interaction was already shown using the yeast two-hybrid system (Spindler et al., 2003) and a pull-down assay (Hoppner et al., 2004). In addition, that interaction was also determined in PC12 cells (Abo Quader, 2013). The author determined that the interaction of Endophilin A3 with SK3 reduced the number of functional SK3 channels, however the reason for that reduction was not determined (Abo Quader, 2013).

Based on all these data, I was interested to further investigate the impact of the interaction of Endophilin A3 on the function of SK3 channels and obtained the following results:

1. The interaction of Endophilin A3 with SK3 channels reduced the channel activity, since the SK3-specific Cs^+ currents measured in Endophilin A3 transfected PC12 cells (-80 ± 60 pA) was reduced compared to currents obtained in control transfected cells (-350 ± 150 pA). These results are in agreement with the results obtained by Abo Quader (2013).
2. Further experiments indicated that the membrane expression of SK3 channels did not change by the interaction with Endophilin A3, since the application of DC-EBIO recovered the SK3 specific current in Endophilin A3 transfected cells (-360 ± 270 pA) to values close to those obtained in control cells (-350 ± 150 pA).
3. My results obtained from the measurement of the intracellular Ca^{2+} concentration simultaneously with the SK3 specific current indicated that the Ca^{2+}-sensitivity of the channel was shifted to the right. Thus, the interaction of Endophilin A3 reduced the Ca^{2+}-sensitivity of the channel thereby leading to less functional channels.
4. The pharmacological effect of DC-EBIO was also tested in control and in Endophilin A3 transfected cells. My results did not show any 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), indicating that the DC-EBIO sensitivity of the channel did not change by the interaction with Endophilin A3.

Based on these results, I could assume that the binding of Endophilin A3 to the N-terminus of SK3 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. It seems that the interaction of Endophilin A3 with SK3 channels may represent another way for tuning the channel activity besides endocytosis and might be an important modulator of signal transduction in neurons. Therefore this interaction seems to be important in controlling some cellular functions where both proteins are colocalized for example in controlling synaptic plasticity and/or enhancing memory encoding and learning.

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