APLP1 and Rab5A Interact with the II-III loop of the Voltage-gated Ca^{2+} -channel $\text{Ca}_v2.3$ and Modulate its Internalization Differently

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Key Words

Protein interaction • Calcium channel internalization
• Calcium homeostasis and channel regulation

Abstract

Background: Voltage gated calcium channels (VGCCs) regulate cellular activity in response to membrane depolarization by altering calcium homeostasis. Because calcium is the most versatile second messenger, regulation of the amount of VGCCs at the plasma membrane is highly critical for several essential cellular processes. Among the different types of VGCCs, the $\text{Ca}_v2.3$ calcium channel and its regulation mechanisms are least understood due to $\text{Ca}_v2.3$'s resistance to most pharmacological agents. **Methods:** In order to study regulation and surface expression of $\text{Ca}_v2.3$, a yeast two hybrid (Y2H) screen with the II-III loop of human $\text{Ca}_v2.3$ as bait, was performed. APLP1, a member of the APP gene family and Rab5A, an endocytotic catalyst were identified as putative interaction partners. The interaction were confirmed by immunoprecipitation. To study the functional importance of the interaction, patch-clamp recordings in $\text{Ca}_v2.3$ stably transfected HEK-293 cells (2C6) and surface biotin endocytosis

assays were performed. **Results:** We are able to show that the II-III loop of the $\text{Ca}_v2.3$ calcium channel binds APLP1 and that this binding promotes internalization of the channel. In addition, Rab5A also binds to the same loop of the channel and exerts an inhibitory effect on APLP1 mediated channel internalization. **Conclusions:** This study identifies a regulation mechanism of $\text{Ca}_v2.3$'s surface expression, which implicates APLP1 as a regulator of calcium homeostasis. Thus APLP1 may play a crucial role in neuropathological mechanisms, which involve modulation of surface expression of voltage-gated Ca^{2+} channels.

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Introduction

Voltage-dependent calcium channels are present on the plasma membrane of excitable cells, where they regulate calcium ion permeability. Calcium ions are the most versatile second messengers and also serve as charge carriers. VGCCs respond to changes in membrane potential and convert cellular electrical excitability into intracellular signaling. Calcium channels are multi-subunit integral membrane proteins, with a large (>250 kD) pore-

forming and voltage-sensing $\alpha 1$ subunit and smaller auxiliary transmembrane $\alpha 2\delta$ and cytoplasmic β subunits [1]. The auxiliary β subunits regulate proteasomal degradation of the $\alpha 1$ subunit [2-4] making them crucial for cellular trafficking and stable surface expression of the $\alpha 1$ subunit. The function of plasma membrane calcium channels can be critically modulated by various signaling pathways, and frequently involves transient or persistent interaction of certain cellular proteins with the $\alpha 1$ subunit. These interactions also tightly regulate the amount of calcium channels expressed on the cell surface. These processes are important because small changes in the number of surface channels can greatly affect cell signaling.

Based on their biophysical and pharmacological properties, VGCCs can be classified into three groups. (i) L-type high voltage-activated (HVA) calcium channels comprising the $\text{Ca}_v 1.1$, 1.2, 1.3, and 1.4 channels, which can be inhibited by dihydropyridines (DHPs) [5, 6], (ii) non-L-type HVA channels $\text{Ca}_v 2.1$ (P/Q-type), $\text{Ca}_v 2.2$ (N-type), and $\text{Ca}_v 2.3$ (R-type) that are sensitive to ω -agatoxin IVA and ω -conotoxin GVIA and SNX 482, respectively [6, 7], and (iii) the low voltage-activated (LVA) T-type Ca^{2+} channel family ($\text{Ca}_v 3.1$, 3.2, and 3.3) [8]. Among the calcium channels, the R-type / $\text{Ca}_v 2.3$ calcium channel has been less explored due to its pharmacological inertness (it's only known peptide inhibitor SNX-482 also antagonizes L- and N-type Ca^{2+} channels at concentrations higher than 300 nM). Under the perforated patch configuration it has been shown in chromaffin cells that 20% of I_{Ca} can be accounted for by toxin-resistant, R-type Ca^{2+} currents [9].

Amyloid precursor proteins form a conserved gene family which, in addition to APP a protein crucial in Alzheimer's disease also includes the mammalian APLP1 and APLP2 (amyloid precursor like proteins). Several different functions of these proteins are suggested, and it is still unclear whether they act as signaling receptors and/or adhesion molecules or if their physiological function may be primarily related to their shedded soluble fragments [10]. While APLP1 is reported to be localized mainly on the cell surface, APP and APLP2 are mostly found in intracellular compartments [11]. APLP1 consists of 650 amino acids and its expression has been reported to be restricted to the nervous system [12]. Interestingly, synthetic peptides corresponding to the cytoplasmic domain of APLP1 and APLP2 have been shown to be phosphorylated by protein kinase C, which also phosphorylates APP [13, 14]. APLP1 also undergoes intramembrane proteolysis like APP and APLP2 [15].

Furthermore, it has recently been shown that APP regulates the expression of $\text{Ca}_v 1.2$ (L-type) calcium channels in striatal and hippocampal GABAergic inhibitory neurons [16]. Additionally, APLP1 has been shown to interact with disabled-1 gene (Dab1), a protein involved in neuronal development and transcription initiation [17] and α_2 -adrenergic receptors [18].

Rab5A is a member of the small G protein superfamily. More than 60 Rab proteins have been identified in mammalian cells. There is ample evidence for Rab protein's role in vesicle trafficking from yeast to humans. GTP dependent Rab proteins are involved in various steps of vesicle trafficking and Rab5 is involved in vesicle transport from the plasma membrane to early endosomes and in early endosome fusion [19]. Recently it was shown that a protein complex consisting of TIP30, endophilin B1 and acyl-CoA synthetase long-chain family member 4, interacts with Rab5A and regulates EGFR endocytosis and signaling [20].

The main focus of this study is to understand the physiological function and regulation mechanisms of Ca^{2+} channels under normal and pathophysiological conditions, including experimentally induced epilepsy [21]. Transcripts of APLP1 changed during pilocarpine-induced epilepsy [22]. As Rab5A and APLP1 were identified by a Y2H screen as potential protein interaction partners of $\text{Ca}_v 2.3$, their function during coexpression with $\text{Ca}_v 2.3$ in HEK-293 cells was further investigated to deepen understanding of the mechanisms related to these interactions.

Materials and Methods

Construction of the bait vector and Y2H

The full length cDNA of $\text{Ca}_v 2.3$ was cloned from fetal brain [23]. The splice variant $\text{Ca}_v 2.3d$ ($\alpha_{1\text{Ed}}$, GenBank # L27745) was subcloned into pcDNA3 vector for transient or stable expression in HEK-293 cells [24]. The $\text{Ca}_v 2.3$ II-III loop was subcloned into pAS2.1 (Clontech-Takara, Saint-Germain-en-Laye, France) at NdeI, EcoRI site using primers:

(forward: GGA ATT CCC ATA TGG ATA ATC TCG CCA ACG CCC, reverse: CCG GAA TTC GCG CAG GTT CAC GAT GTA GTG GC), and this vector was used as bait.

For the Y2H, the MATCHMAKER system from Clontech was used (now Clontech/Takara, Japan) following manufacturer's instructions. The clones were isolated from a pre-transformed human brain cDNA library (Clontech, number HY4004AH).

Plasmid constructs

The epitope tagged clones of APLP1 and Rab5A were constructed from cDNA clones obtained from RZPD cDNA

library. PCR was performed with *pfu* polymerase using the following primer pairs. APLP1-FL (forward: ccc aag ctt ccc gcc gcc gcc cgc aag ggc cgg gac atg, reverse: ccg ctc gag cct ggt cgt tcc tcc agg aag c), APLP1-IS (forward: ccc aag ctt ccc gcc gcc gcc atg ctg agg gcc aag atg gac, reverse: act cga gcc ccc gta ggg ctt ctt cct g), APLP1-IS-CID (forward: ccc aag ctt ccc gcc gcc gcc atg ctg agg gcc aag atg gac, reverse: ccg ctc gag cct ggt cgt tcc tcc agg aag c), Rab5A (forward: ccc aag ctt ccc gcc gcc gcc atg gct agt cga ggc gca aca aga cc, reverse: act cga gcc gtt act aca aca ctg att cct ggt tgg ttg tgt gg). The amplified regions were inserted at the HindIII / XhoI site in pcDNA myc/his B vector with a c-terminal myc tag. C-terminal Flag tag of II-III loop was performed as described previously [25]. Briefly, full length II-III loop was amplified from the Cav2.3d-pcDNA3 vector with forward primer containing the NotI site (atg cgg ccg ccc acc atg gat aat ctc gcc aac gcc cag), and the reverse primer containing a SalI site (acg tgc acg geg cag gtt cac gat) and subcloned into pIRES-hrGFP-1a vector at NotI - SalI site. The constructs were verified by sequencing.

Cell culture and transfection

HEK 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics penicillin / streptomycin. 0.25% trypsin was used to detach the cells and was split in the ration of ~1:10. Ca_v2.3 alpha subunit stable transfection of HEK 293 cells (generation of 2C6 cells) is described in Pereverzev et al, 1998 [26]. The 2C6 cells express only the human Ca_v2.3d, a fetal brain splice variant with an extended carboxy terminal insertion [23]. No auxiliary subunits were cotransfected. HEK 293T and 2C6 cells were cultured as described above, in addition 1 mg/ml geneticin was added to 2C6 cells. For freezing the cells, the complete growth medium was supplemented with 10% (v/v) DMSO and stored overnight at -80°C and transferred to liquid nitrogen. FuGene HD (Roche Diagnostics, Mannheim / Germany) was used for transient transfection following the manufacturer's instructions. For Myc and Flag immunoprecipitations, the Flag tagged II-III loop of the channel and Myc tagged protein partner (APLP1 or Rab5A) were transfected in HEK 293T cells and lysed after 72 hours of transfection. For surface biotin endocytosis, the cells were taken for biotinylation after 48 hours of transfection.

Immunoprecipitation

Cells were briefly rinsed with PBS and lysed (CellLytic M Cell Lysis Reagent, C2978, Sigma) on ice for 30 mins. The lysates were centrifuged at 12000 rpm for 10 mins at 4°C and the supernatants were quantified using BCA assay kit (Thermo Fisher Scientific, Bonn) and equal amount of proteins were used for immunoprecipitation with appropriate agarose beads at 4°C. Immunoprecipitation was carried out for 2 hours for anti-FLAG beads (FLAGIP KIT, Sigma-Aldrich, Munich, Germany), 1 hour for anti-Myc beads (Anti c-myc IP kit Sigma-Aldrich, Munich, Germany), and overnight for NeutrAvidin beads (Thermo Fisher Scientific, Bonn). Beads were then centrifuged at 1000 rpm for 1 min at 4°C and washed according to manufacturer's instructions at 4°C. Proteins were eluted with Laemmli buffer except for those incubated with Anti-FLAG beads which were eluted with FLAG peptide.

Whole-cell patch-clamp

Ca_v2.3 stably transfected HEK 293 cells (2C6 cells) were plated at very low density on 0.1% gelatin coated cover slips, one day before patch-clamping. Borosilicate glass capillaries of 2 mm thickness were pulled using a horizontal pipette puller (P-97 micropipette puller, Sutter instruments) in such a way that it has a resistance of 3 to 6 MΩ after filling with the internal standard solution (130 mM CsCl, 5 mM oxaloacetate, 5 mM creatine, 5 mM pyruvate, 0.02 mM EGTA, 10 mM HEPES, pH 7.3 and 272 mosmol / kg). The external standard solution or bath solution used contained 5 mM CaCl₂, 120 mM NaCl, 10 mM HEPES, 5 mM KCl, 1 mM MgCl₂, 20 mM tetraethylammonium chloride, pH 7.35, 305 mosmol / kg. Single cells were chosen for patch-clamp recordings. Suction was applied using a mouth-piece in order to break into the cell, and the currents were measured once a giga ohm seal was formed. From a holding potential of -90 mV, a test potential to +30 mV for 400 ms was applied using the EPC-9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Currents were filtered with a three-pole analog Bessel filter set as a cut-off (-3dB) at 10 kHz. Data was analyzed with PulseFit software.

Surface biotinylation

Ca_v2.3 stably transfected HEK 293 cells (2C6) were plated on poly-d-lysine (P6403, Sigma) coated 100 mm tissue culture dishes. The cell surface proteins were biotinylated (Pierce EZ-Link Sulfo-NHS-SS-Biotin) on ice for half an hour in the dark. The biotin was washed off with ice cold 1x PBS ++ (1x PBS supplemented with 0.9 mM CaCl₂ and 0.33 mM MgCl₂) and the excess biotin was quenched with PBS containing 0.1% BSA. The surface biotin-labeled cells were allowed to undergo endocytosis at 37°C in 10% DMEM for 30 mins. After 30 mins DMEM was washed off and the surface biotin was stripped off using MESNA buffer to be able to identify the internalized portion of the channel. The internalized channel was detected by the accumulation of the biotin labeled cargo proteins within the cells, which were protected from reduction by MESNA. A portion of the cells, which were biotinylated, quenched by 0.1 % BSA and washed (but not MESNA-treated) represented the positive control and another portion which were biotinylated and reduced with MESNA without allowing internalization represented the negative control. The cells were then lysed with the buffer containing 20 mM Tris, pH 7.4, 5 mM EDTA, 135 mM NaCl, 0.5% (v/v) Nonidet P-40, and 10% (w/v) sucrose, quantified and equal amount of proteins were immunoprecipitated with NeutrAvidin beads. Internalized channel proteins were detected with polyclonal channel antibody named as 195A raised in-house [for description of antibody production see [27]].

Quantification of immunoblot

The amount of protein expressed in different samples in an immunoblot was quantified using ImageJ software. After developing the chemiluminescent immunoblot, it was scanned and exported as an image file. The intensity of the protein band was quantified after background subtraction.

Statistical Analysis

In all experiments the data are expressed as the mean $\pm$ SEM. Overall statistical significance was determined by analysis of variance. *p* values below 0.05 were considered significant and those below 0.01 were considered highly significant.

Results

Identification of interaction partners for the cytosolic II-III linker of *Ca_v2.3*

The cytosolic II-III loop of the non-L-type *Ca_v2* subfamily is crucial for excitation-secretion coupling [28]. To determine the interacting proteins, the II-III loop of *Ca_v2.3* was used as bait, and a human brain cDNA library was screened for interaction proteins using the yeast-two-hybrid technology similar as recently performed for the C-terminus [29]. During the first screen, 195 colonies were obtained that survived on synthetic media lacking tryptophane, leucine, and histidine. After a four-fold selection including testing for adenine auxotrophy, cells from 141 colonies survived. Out of these a pool of 66 colonies were proven to be beta-galactosidase positive. After PCR-amplification and subcloning, 14 genes of interest were left. Of these positive interacting candidates, Rab5A and APLP1 are reported here. Both candidates were selected to show potential biochemical as well as functional interaction with *Ca_v2.3*, under the hypothesis that variation in endocytosis may influence surface expression of *Ca_v2.3* and thus excitability of hippocampal neurons [30]. Recently, it was demonstrated that surface expression of APLP1 varied during experimentally induced epilepsy [22], which may be mediated by regulating *Ca_v2.3* surface expression. Rab5A was selected, because it is known as a marker protein for early endosomal and recycling compartments.

Interaction of Rab5A with *Ca_v2.3*

Rab family proteins regulate vesicular trafficking pathways, behaving as membrane-associated molecular switches. Rab5A is present on the cell membrane, early endosomes and melanosomes. It supports the fusion of endocytotic vesicles and the formation and transport of early endosomes [31]. Immunoprecipitation of Rab5A and II-III loop proteins was performed using anti-MYC agarose beads. Immunoblotting was performed to detect the precipitated proteins with FLAG antibody (Fig. 1a). The II-III loop was detected in the experimental lane at a higher size than the expected size of 54 kDa due to its charged nature. And also a small amount of the II-III loop bound to the MYC beads in the absence of Rab5A

Fig. 1. Rab5A interaction with II-III loop: (a) Immunoblot (IB) with FLAG antibody and MYC immunoprecipitation (IP). The input or the whole cell lysate shows the expression of II-III loop in 293 T cells and the MYC-IP shows the immunoprecipitation of II-III loop. In lane 2 of the IP, there is a non-specific binding of II-III loop to the MYC beads in the absence of MYC tagged Rab5A. (b) The non-specific band is significantly lower in intensity compared to the specific band as quantified by ImageJ from three independent experiments. The data is represented as mean $\pm$ SEM. (c) Immunoblot with MYC antibody and immunoprecipitation with MYC beads. The input is the whole cell lysate showing the expression of Rab5A in 293T cells and the MYC-IP shows the precipitation of Rab5A. Note, on the left panel (input) higher molecular weight proteins are unspecifically stained, which are not precipitated by anti-myc beads.

was seen, which was significantly much lower (0.35 ± 0.10) than the specific band as obtained from three independent experiments (Fig. 1b). The blot was stripped and re-probed with anti-myc antibody and the 24 kDa Rab5A band was observed, demonstrating the expression and appropriate immunoprecipitation of Rab5A (Fig. 1c). Thus II-III loop of *Ca_v2.3* channel binds to Rab5A. Since

Fig. 2. APLP1-IS binds to II-III loop: (a) Immunoblot (IB) with MYC antibody, immunoprecipitation (IP) with FLAG beads. The input shows the expression of APLP1-IS in 293T cells and the FLAG-IP shows precipitation of APLP1-IS. Note the increased number of immunopositive bands (different sizes) on the left panel, when APLP1-IS-myc was transfected. The pattern of smaller sized proteins was routinely seen and also reported in the literature. After immunoprecipitation (right panel), only the largest protein was precipitated, because it was the most dominant one. After longer exposition, the smaller ones could sometime be visualized. Lane 2 and 4 on the left panel show unspecific bands, which were never precipitated. (b) Immunoblot with FLAG antibody, immunoprecipitation with FLAG beads. The input shows the expression of II-III loop in 293T cells and the FLAG-IP shows the precipitation of II-III loop.

the latter is an endocytotic protein, this binding indicates possible involvement in endocytosis of the channel.

APLP1 binds to Ca_v2.3 II-III loop

APLP1, a member of the highly conserved amyloid precursor protein family [32] mainly expressed in neural tissue [33] is a novel putative interaction partner identified from the Y2H screen in which the Ca_v2.3 II-III loop was used as bait. This screen identified the region between 999-1899 bp (Accession: BC012889) of APLP1 (referred here as APLP1-IS) as the site that interacts with the II-III loop. This consists of a part of the extracellular region, the transmembrane domain, and a part of the cytosolic domain, predicted to be 6 aa in length, which represents the minimum length for possible protein-protein interaction. Therefore, a second clone of APLP1-IS was constructed which additionally consisted of the complete cytosolic

Fig. 3. APLP1-IS-CID interaction with II-III loop: (a) Immunoblot (IB) with MYC antibody, immunoprecipitation (IP) with FLAG beads. The input shows the expression of APLP1-IS-CID in 293T cells and the FLAG-IP shows the precipitation of APLP1-IS-CID. (b) Immunoblot with FLAG antibody, immunoprecipitation with FLAG beads. The input shows the expression of II-III loop in 293T cells and the FLAG-IP shows the precipitation of II-III loop.

region and is here referred to as APLP-IS-CID. FLAG immunoprecipitation of either of these APLP1 peptides along with the FLAG-tagged II-III loop was performed and eluted with FLAG peptide. The APLP1 peptides were detected with MYC antibody. A specific band for both the APLP1-IS (Fig. 2a) and APLP1-IS-CID (Fig. 3a) clones were detected at higher molecular weights compared to the theoretical size of 33 and 36 kDa respectively. This is probably due to the presence of a hydrophobic transmembrane region of APLP1. The blots were stripped and re-probed with anti-FLAG antibody and the II-III loop was detected (Fig. 2b & 3b). This confirmed the expression and appropriate immunoprecipitation of the channel loop showing that both APLP1 peptides interact with Ca_v2.3 channel.

Internalization of Ca_v2.3 channel

Since proteins of the APP gene family have been associated in many cellular functions including neuroprotection and vesicle transport, it was hypothesized that binding of APLP1 to Ca_v2.3 might influence the internalization of the channel. To test this hypothesis, a biotin endocytosis assay was performed. The surface

proteins of 2C6 cells were biotinylated (surface biotinylation) and channel proteins that underwent endocytosis were detected using a channel specific antibody. The $\alpha 1$ subunit of the $\text{Ca}_v2.3$ channel from 2C6 cells was observed at ~ 263 kDa. Endocytosis was assayed at different time points. Maximum channel internalization was observed at 30 mins (not shown).

APLP1 enhances the internalization of $\text{Ca}_v2.3$

With the objective of studying whether APLP1 modulates internalization of $\text{Ca}_v2.3$ calcium channels, a biotin endocytosis assay was performed in 2C6 cells transfected with either APLP1-IS or APLP1 full-length (FL). The biotinylated channel protein was detected with polyclonal channel antibody (195A) (Fig. 4). The empty vector transfected 2C6 cells showed a very scarce internalization (lane 3) and the APLP1-IS transfected cells (lane 4) also showed basal channel internalization similar to the empty vector. Interestingly, cells transfected with APLP1-FL (lane 5) exhibited strikingly higher channel internalization, which may be explained by the possibility of the full length protein to form dimers [11, 34]. The increase in intensity of the internalized band was significantly higher than the mock control as quantified from four independent experiments. This led to the conclusion that the full-length APLP1 including the extracellular part and the cytosolic tail increases the internalization of the channel.

Rab5A and channel endocytosis

Upon expressing Rab5A with APLP1-FL in 2C6 cells, there was a visible increase in channel internalization (Fig. 4, lane 6), and with expression of Rab5A alone (lane 7), there was an increase, however quite modest compared to the basal internalization (Fig. 4). However, both increases were not significant compared to the basal rate of the mock control. The most important observation was that channel internalization in the presence of APLP1-FL was significantly higher than in the mock control, and interestingly cotransfection of APLP1-FL with Rab5A significantly reduced $\text{Ca}_v2.3$ internalization (Fig. 4). Even Rab5A only reduced internalization significantly, possibly because APLP1 is expressed endogenously. These results show that APLP1 is majorly responsible for channel endocytosis and Rab5A restrains APLP1 mediated channel endocytosis. Additionally, the blot was stripped and re-probed with Myc antibody to inspect endocytosis of APLP1-FL. The APLP1-FL band was detected at the expected size of 100 kDa. Cells transfected with APLP1-FL alone, exhibited APLP1 endocytosis, however in cells

Fig. 4. Internalization of $\text{Ca}_v2.3$: (a) Immunoblot (IB) with 195A antibody, immunoprecipitation (IP) with NeutrAvidin beads. The blot shows the 1: Positive control, 2: negative control, 3: channel internalized with mock control, 4: with APLP1-IS, 5: with APLP1-FL, 6: with APLP1-FL + Rab5A and 7: with Rab5A. The input shows the whole-cell lysate in the same order as above. Note, the $\text{Ca}_v2.3$ detected by the 195A antibody was seen as a weak double band, which may result from proteolytic processing of the carboxy terminus as reported for the skeletal muscle and the cardiac L-type Ca^{2+} channel. (b) ImageJ quantification of biotin endocytosis of $\text{Ca}_v2.3$. APLP1-FL expression shows a highly significant increase in the internalization of the channel compared to mock controls. Rab5A caused a moderate increase in channel internalization but significantly decreased APLP1 mediated internalization of the channel. Statistics were performed from four independent experiments. The data is represented as mean $\pm$ SEM. * symbol denotes statistical significance and ** denotes high significance.

in which Rab5A was co-expressed APLP1 endocytosis was drastically increased (Fig. 5). These results illustrate that Rab5A has an inhibitory effect on APLP1 mediated internalization of the channel by increasing APLP1 endocytosis.

Fig. 5. APLP1 endocytosis with Rab5A: (a) Immunoblot (IB) with MYC antibody, immunoprecipitation (IP) with the NeutrAvidin beads. The blot shows IP of biotinylated proteins, lane 1: Positive control, 2: negative control, 3: channel internalized with mock control, 4: with APLP1-IS, 5: with APLP1-FL, 6: with APLP1-FL + Rab5A and 7: with Rab5A. Lanes 5 and 6 show the endocytosis of APLP1 without and with Rab5A expression respectively. Note, the naming of the individual lanes refers to the original blot in Fig. 4a. This blot had been stripped for reprobing with MYC antibody. (b) ImageJ quantification of lanes 5 and 6, taken from two independent experiments. The APLP1 endocytosis is increased dramatically with Rab5A expression.

Ca_v2.3 current density with APLP1

As previously mentioned, current density measurement using whole-cell patch clamp recording is a measure of the amount of active channels on the plasma membrane. Since APLP1 was shown to be involved in removal of channels from the cell surface, we investigated whether APLP1 expression affected current density. For this, 2C6 cells were transfected with APLP1-FL-GFP or control GFP. Maximum inward currents were acquired and the ratio of peak inward currents to that of cell capacitance provided the current density. The mean

Fig. 6. Current density of Ca_v2.3 with APLP1: 2C6 cells were transfected with APLP1-FL. Single cells were chosen for patch-clamp recordings. The currents were elicited from a holding potential of -90 mV by a test potential to +30 mV for 400 ms. (a) The open bar indicates the current density (peak inward current divided by the cell capacitance) of Ca_v2.3 with APLP1-FL-GFP and the grey bar indicates the same with mock vector. The data is represented as mean ± SEM. * symbol denotes statistical significance. (b) Mean current trace (n = 4) of Ca_v2.3 with APLP1-FL. (c) Mean current trace (n = 5) of Ca_v2.3 with GFP-control. (d) Mean current traces of Ca_v2.3 with APLP1-FL and GFP-control super-imposed.

current densities of $\text{Ca}_v2.3$ with APLP1-FL and GFP control in 2C6 are illustrated (Fig. 6a). There was no difference in current density for shorter APLP1 peptides (data not shown). Upon expressing APLP1-FL, there was a statistically significant decrease in current density. Mean current densities of APLP1-FL and mock are -6.3 ± 1.3 pA/pF and -12.6 ± 1.4 pA/pF respectively. The current traces of APLP1-FL and GFP mock vector are shown in Fig. 6b, 6c and the superposed traces of normalized currents in Fig. 6d. Thus in the presence of APLP1-FL, mean peak inward currents are significantly decreased and current inactivation kinetics changed. Especially the decrease in current density of $\text{Ca}_v2.3$ channels in the presence of APLP1-FL shows that APLP1 modulates the calcium current, which would be in agreement with results obtained from the biotin endocytosis assay, if endocytosis has reduced surface expression of $\text{Ca}_v2.3$.

Discussion

VGCCs are regulated by channel gating, auxiliary subunits, calcium sensors and membrane voltage. In addition, they also are subjected to a variety of regulatory mechanisms mediated by signaling proteins, which may regulate the channel by direct interaction or via scaffold proteins. Protein interaction with the channel may be constitutive or transient. Such regulatory interactions can lead to modulation of channel gating, assembly, trafficking or response of the channel to changes in cellular signaling events. Here, APLP1 and Rab5A, two novel interaction partners of the II-III loop of the $\text{Ca}_v2.3$ calcium channel, were identified and functionally investigated. The results show that APLP1 together with Rab5A is involved in regulation of $\text{Ca}_v2.3$ channel expression on the cell surface.

Endocytosis of $\text{Ca}_v2.3$

Interaction of APLP1 with $\text{Ca}_v2.3$ remarkably increased the amount of channels internalized in $\text{Ca}_v2.3$ stably transfected cells. It is notable that the full-length APLP1 protein (APLP1-FL) alone caused a pronounced increase in channel internalization and not the truncated version lacking the extracellular region of APLP1 (APLP1-IS, Fig. 4). This suggests that the extracellular region of APLP1 and probably the signal it receives is important for endocytosis of the $\text{Ca}_v2.3$ channel.

In coherence with these results, the current recordings using patch-clamp on 2C6 cells expressing APLP1-FL showed reduced current density of the channel

(Fig. 6a), indicating that the number of active channels on the cell membrane is reduced due to APLP1 mediated internalization. Coexpression of APLP1-FL additionally affected the inactivation kinetics of inward currents. This was also observed in 2C6 cells cotransfected with APLP1-IS, the truncated construct, which did not affect mean current density.

One could hypothesize that the extracellular domains have to be in full length to enable dimerization of the APLP1 complex [34]. Previously, it has been shown that APLP1 binds to α_{2A} -adrenergic receptors and plays a role in regulation of α_{2A} -adrenergic receptor trafficking [18]. Similarly, here APLP1-mediated internalization of $\text{Ca}_v2.3$, may act as a negative feed-back mechanism of the channel, implicating APLP1, in such a novel regulatory role. It can further be hypothesized that by internalizing active channels, APLP1 contributes to the regulation of calcium homeostasis in neuronal cells. The activation of $\text{Ca}_v2.3$ channels and subsequent calcium influx may recruit APLP1 to the channel. Actively recruited APLP1 could reduce the probability of further calcium entry by triggering internalization of the channel. Such a mechanism can physiologically be attributed to neuroprotection. Interestingly, a report demonstrated that expression of APLP1 mRNA is down regulated in pilocarpine-induced epileptic rats [22]. In such pathological conditions, due to APLP1 down-regulation and non-availability of APLP1 for channel endocytosis, active $\text{Ca}_v2.3$ on the plasma membrane could lead to elevated intracellular calcium levels, which may contribute considerably to pilocarpine-induced epilepsy. This view is supported by the observation that $\text{Ca}_v2.3$ knockout mice are neuroprotected after kainate injection when compared to wild type mice [30]. Taken together, this provides a novel insight into the mechanism of feed-back regulation of $\text{Ca}_v2.3$ and reveals yet another unknown function of APLP1.

Rab5A and channel internalization

The small GTPase Rab5A is a well known endocytotic regulator. A large body of evidence has been recently accumulated in support of a role for Rab proteins in vesicle trafficking, providing crucial insights into how Rab proteins may be involved in the distribution of ion channel proteins between different compartments within the cell and the plasma membrane [19]. Rab5 regulates trafficking of cystic fibrosis transmembrane conductance regulator (CFTR) from the plasma membrane to early endosomes [35]. In the current study, Rab5A has been shown to interact with $\text{Ca}_v2.3$ (Fig. 1), causing a slight

increase in channel internalization. Intriguingly, Rab5A significantly decreased the APLP1 mediated channel internalization and more strikingly, Rab5A dramatically increased the endocytosis of APLP1 (Fig. 5). This is in agreement with previous reports that APP family proteins, co-localize with Rab5A [36, 37]. This process of APLP1 endocytosis in presence of Rab5A limits the availability of APLP1 for channel internalization and thus creates a balance in calcium homeostasis against APLP1, indicating that APLP1 mediated internalization is crucial in maintaining calcium homeostasis in neuronal cells. Thus Rab5A is involved in this mechanism by negatively influencing the process of APLP1 mediated endocytotic trafficking of the channel.

Conclusion

Plasma membrane targeting of calcium channels has been studied considerably, however the removal of channels from the membrane is only recently coming into focus. Altier et al. [38] and Green et al. [39] have independently shown the internalization of N-type and L-type calcium channels respectively. The N-type calcium channel binds to ORL1 (opioid receptor like receptor 1) receptor and activation of ORL1 triggers the internalization of N-type calcium channels [38]. The II-III loop of the L-type calcium channel, Ca_v1.2, binds to eIF3e (eukaryotic initiation factor 3 subunit e), a tumor suppressor, which mediates activity dependent internalization and trafficking of the channel [39]. Most interesting is the observation that the auxiliary β subunit influences proteasomal degradation of Ca_v2.2 / N-type and Ca_v1.2 / L-type Ca²⁺ channel [2, 3]. Concordantly, it could be hypothesized that

APLP1 enhances the internalization of Ca_v2.3 calcium channel which is negatively influenced by Rab5A. It is interesting to note that a member of the APP family interacts directly with a calcium channel and cause its internalization. This could function as a negative feedback mechanism, controlling the expression of the channel at the plasma membrane, thereby establishing calcium homeostasis. Thus APLP1 mediated internalization may represent an important neuroprotective mechanism in pathophysiological conditions such as epilepsy involving Ca_v2.3. Further studies in this line could possibly establish APLP1 and its interaction with Ca_v2.3 as a novel therapeutic target in neurodegenerative diseases.

Abbreviations

APLP (Amyloid Precursor Like Protein); APP (Amyloid Precursor Protein); II-III loop (Cytosolic linker between the 2nd and 3rd transmembrane domains); EGFR (Epidermal growth factor receptor); EDTA (Ethylene-diaminetetraacetic acid); HVA (High Voltage Activated); PBS (Phosphate Buffered Saline); MESNA (Sodium 2-Mercaptoethanesulfonate); VGCC (Voltage gated calcium channels); Y2H (Yeast Two Hybrid).

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