Ca_v2.3 Ca²⁺ Channel Interacts with the G1-subunit of V-ATPase

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Key Words

Protein interaction • Secretion-pore • Synaptic vesicle proteins • V-ATPase • G1-subunit • R-type • Calcium channel

Abstract

Background: Calcium channels are essential in coupling action potential to signal transduction in cells. There are several types of calcium channels, which can be pharmacologically classified as L-, N-, P/Q-, R- and T-type. But molecular basis of R-type channels is less clearly understood compared the other channel types. Therefore the current study aims at understanding the molecular functions of R-type calcium channels by identifying interaction partners of the channel. **Methods:** In order to do so, a yeast two hybrid (Y2H) screen, with carboxy terminus of α_1 subunit of the channel, as the bait, was performed. G1 subunit of v-ATPase was identified as a putative interaction partner of human Ca_v2.3 by using the Y2H screening. The interaction was confirmed by immunoprecipitation. To study the functional importance of the interaction, bafilomycin A₁, a potent and specific inhibitor of v-ATPase was used in patch-clamp recordings in Ca_v2.3 stably-transfected HEK-293 cells

(2C6) as well as in electroretinography of the isolated bovine retina expressing R-type Ca²⁺ channels. **Results:** G1 subunit of v-ATPase interacts with C-terminal tail of Ca_v2.3 and bafilomycin A₁ reduces Ca_v2.3 mediated calcium currents. Additionally peak I_{Ca} is inhibited in retinal signal transduction when recorded as ERG b-wave. **Conclusions:** The results suggest that v-ATPase interacts physically and also functionally with Ca_v2.3. This is the first demonstration of association of Ca_v2.3 C-terminus with a protein complex which is involved in transmembrane signalling.

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Introduction

Influx of calcium through VGCC triggers secretion and neurotransmitter release, both of which are further modulated by hormone receptors and second messengers. Further, signalling through VGCC involves direct interactions of cytosolic channel segments with target proteins as was shown for auxiliary subunits of the calcium channels, for trimeric G-protein subunits and for synaptic

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vesicle proteins [1-8]. For the Ca_v2.3 II-III loop, Hsp70 was identified as a binding partner, which may act as a scaffold protein and connect protein kinase C to Ca_v2.3 phosphorylation [9]. Besides classical biochemical investigations, the large scale Y2H screening has become crucial for the identification of novel protein interactions and even proteome-wide interaction maps [10], as well as quantitative proteomic approaches have identified the nano-environment of voltage-gated Ca²⁺ channels [11]. Such approaches are needed and provide important information for the functional involvement of the Ca_v2.3 Ca²⁺ channel and its interaction partners in molecular processes of hippocampal excitability [12, 13] and its disturbance in convulsive diseases [14].

For Y2H, the C-terminus of the Ca_v2.3 was chosen as bait. In high voltage gated calcium channels, this region is important for calcium dependent inactivation [15-17] and transcription regulation [18]. For inherited forms of epilepsy, EFHC1 was identified to interact with the C-terminus of Ca_v2.3. The disturbance of such an interaction by mutations in EFHC1 causes juvenile myoclonic epilepsy [19]. In the Y2H screen, several interaction proteins including calmodulin-2 and the G1 subunit of v-ATPase were identified. G2 subunit of v-ATPase has been reported to interact with L-type Ca²⁺ channels [20] leading to the suggestion that the interaction of VGCC with a multi-subunit protein complex as the v-ATPase is mediated through small protein subunits. Here we report the functional interaction of G1 with Ca_v2.3 as was investigated in 2C6 cells and in the isolated and superfused bovine retina.

Materials and Methods

Materials

The mammalian expression vectors, pcDNA 3.1 Myc/His B (V800-20) and pIRES-hrGFP-1a (#240031), were purchased from Invitrogen and Stratagene, respectively. The FLAG immunoprecipitation kit (FLAGIPT1) and other chemicals were from Sigma (München, Germany). The anti-myc antibody was purchased from Invitrogen (Karlsruhe, Germany; catalogue number R950-25) and was diluted (1:5000) as recommended by the company. Bafilomycin A₁ was purchased from Sourcon-Padana (88899-55-2).

Construction of the bait vector and Y2H

The full length cDNA of Ca_v2.3 was cloned from fetal brain [21]. It corresponds to the splice variant Ca_v2.3d (α_{1Ed}, GenBank # L27745) which was subcloned into the pcDNA3 vector for transient or stable expression in HEK-293 cells [22]. The cDNA encoding the full length C-terminus was PCR ampli-

fied from the pcDNA3-Ca_v2.3d vector with the oligonucleotides 5'-AAA GTC GAC TGG ACA ACT TTG AGT ACC TG-3' and 5'-AAC TGC AGG CGG AGG GGG GAG GTACCT CTAGCA-3' and cloned at SalI, PstI site of TOPO2.1 (Invitrogen). When sequenced it contained a silent mutation (T-5430 in L27745 was changed into C). The Ca_v2.3 C-terminus was subcloned into pGBKT7 (Clontech-Takara, Saint-Germain-en-Laye, France) at Sal I, Pst I site, and 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).

Construction of vectors for immunoprecipitation

The full length cDNA encoding the Ca_v2.3 C-terminus was PCR amplified from the Ca_v2.3d-pcDNA3 vector [22] with the oligonucleotides 5'-AAA GCG GCC GCA ACC CGC CGC CGC CAT GGA C-3', and 5'-AAA GTC GAC CGC ATT TGT CAT CTT CTT CCG T-3', and cloned at NotI, Sal I site of pIRES-hrGFP-1a.

The v-ATPase G1 was PCR amplified from the G1-containing yeast clone-6 with the oligonucleotide 5'-TTT-AAG-CTT-CCC-GCC-GCC-GCC-ATG-GCT-AGT-C-3' and 5'-TTT-CTC-GAG-CCT-CCA-TTT-ATG-CGG-TAG-TTT-TCA-TGG-ATT-3' and subcloned into pcDNA3.1/myc-His at HindIII, XhoI site. All clones were verified by sequencing.

Cell culture and immunoprecipitation

HEK-293T cells were cultured in DMEM containing 10% FBS and 100 mg/ml Penicillin and Streptomycin in 37°C incubator with 5% CO₂. The FLAG-tagged C-terminus of Ca_v2.3 and Myc-tagged G1 subunit of v-ATPase were transfected with FuGENE HD (Roche, Mannheim, Germany) in HEK-293T cells. After 72 hours of transfection, the cells were lysed and FLAG-immunoprecipitation was done using FLAGIPT1 kit as per manufacturer's protocol [9]. The precipitated proteins were separated on SDS-PAGE and western blot was done using appropriate antibodies and detected with chemiluminescence detection (ECL, RPN2209, GE Healthcare).

Voltage-clamp recordings

Membrane currents from 2C6 cells were recorded in the whole-cell configuration of the patch clamp [23, 24]. These cells were voltage-clamped at -90 mV using the EPC-9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). The values for the cell capacitance varied between 10 pF to 45 pF. Currents were filtered with a three-pole analog Bessel filter set as a cut-off (-3dB) at 10 kHz. Patch clamp electrodes were pulled from thick wall borosilicate glass using a Sutter P97 horizontal puller (Sutter Instrument Company, Novato, CA, USA). They had a resistance of 3 MΩ to 7 MΩ when filled with the electrode solution containing (in mM) 130 CsCl, 5 oxaloacetate, 5 creatine, 5 pyruvate, 0.02 EGTA, 10 HEPES adjusted to pH 7.35 with CsOH (osmolality: 272 mosmol/kg). The bath solution contained (in mM) 5 CaCl₂, 160 N-methylglucamine, 10 HEPES, 5 KCl, and pH 7.35 with HCl (osmolality: 305 - 320 mosmol/kg). All experiments were performed at room temperature. Gravitation

Fig. 1. Constructs used for screening and for expression studies. Vector constructs for screening a human brain library by Y2H. C-terminus of $\text{Ca}_v2.3$ was cloned into pGBKT. Vector constructs of FLAG-tagged C-terminus from $\text{Ca}_v2.3$. C-terminus was subcloned into pIRES-hrGFP-1a vector. It was fused in frame to a 3x FLAG-epitope for immunoprecipitation.

driven perfusion was performed using a precision perfusion controlling system and a round cover slip chamber (RC-25, 13 mm, Warner Instruments, Hamden, CT, USA). Bafilomycin A_1 was dissolved in DMSO and prepared as a 1000-fold stock solution.

ERG recordings from the isolated superfused bovine retina

The isolated bovine retina was perfused with a nutrient solution either containing the drug of interest or the solvent carrier as control [25-27]. In the local slaughterhouse, eyes were removed from a calf and immediately stored in an empirically optimized nutrient solution consisting (in mM): 120 NaCl, 2.0 KCl, 0.15 CaCl_2 , 0.1 MgCl_2 , 1.5 NaH_2PO_4 , 13.5 Na_2HPO_4 , and 5 glucose, pH - 7.8. The eyes were hemisected at the junction of sclera and cornea followed by removal of lens and vitreous body from the posterior eyecup. Circular pieces were obtained from the posterior segment, subsequently, the retina was detached from the underlying pigment epithelium by gentle shaking. Retina segments were transferred to a perfusion chamber in an electrically and optically insulated box [25]. The whole preparation was carried out under dim

red illumination. Prior to experiments the retina was continuously superfused by the nutrient solution at a flow rate of 1 ml/min. Temperature was kept constant at 30°C, and the perfusion medium was pre-equilibrated and saturated with oxygen.

Following retinal dark adaptation, the ERG was elicited by a single white flash (6.3 mJ) at intervals of 5 min. The ERG was amplified and bandpass limited between 1 and 300 Hz. The signal was AD converted and stored using a PC-based signal acquisition and analysis system.

NiCl_2 was added from a 10 mM stock solution. Drug application was initiated after the b-wave had reached stable amplitude, followed by a recovery period of at least 60 min. The b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave.

Data analysis and curve fitting

Current amplitudes and kinetics were recorded as reported [23]. The data are expressed as mean $\pm$ SEM, and n is the number of cells used. Statistical significance was analysed using Student's t -test and levels of $p < 0.05$ were considered as statistically significant.

Fig. 2. Immunoprecipitation of FLAG-tagged C-terminus and c-myc-tagged G1 subunit. After expression of FLAG tagged $Ca_v2.3$ C-terminus and myc tagged G1, v-ATPase, cell lysate from 293T cells were extracted and precipitated by anti-FLAG beads. The proteins were eluted by an excess of FLAG-peptide (150 ng/ml) and were separated by SDS-PAGE. After western blotting, anti-c-myc was used as the primary antibody (panel A1 and A2) or after stripping the blot it was re-probed with anti-FLAG (panel B). This figure shows the results from one out of three experiments.

Results

Identification of interaction partners of $Ca_v2.3$ C-terminus

The C-terminus of VGCC is important for physiological feedback regulations *in vivo* [15, 28, 29]. To determine the interacting proteins, C-terminus of $Ca_v2.3$ was the bait (Fig. 1). A human brain cDNA library was screened for interaction proteins using Y2H. During the first screen, 72 colonies were obtained that survived on synthetic media lacking tryptophane, leucine, and histidine. After a four-fold selection including the testing for adenine auxotrophy, cells from 19 colonies survived. Out of these, a pool of 11 colonies were proven to be beta-galactosidase positive. After PCR-amplification and subcloning, 9 genes of interest were left. Of these positive interacting candidates, the G1 subunit of v-ATPase is reported here.

Interaction of v-ATPase G1-subunit with the C-terminus of $Ca_v2.3$

To confirm the interaction, MYC tagged G1 subunit of v-ATPase, and FLAG tagged C-terminus of $Ca_v2.3$ were transfected in HEK-293T cells and immunoprecipitated with FLAG beads (Fig. 2). The proteins were quantified by the BCA method and equal amounts of proteins were used for immunoprecipitation. The proteins were eluted with 150 ng /ml of FLAG peptide. After 10 min exposure the band for G1 was detected but upon longer exposure a faint band in control lane was seen which suggests that there is a small amount of non-specific binding of G1 protein to FLAG beads, which, however, is markedly lower than the specific binding, from three independent experiments. Thus, specific protein binding in the presence of $Ca_v2.3$ C-terminus was 7.3 ± 1.3 fold (n = 3) higher than the unspecific binding,

Fig. 3. Bafilomycin A₁ reduces the activity of Ca²⁺ channels in Ca_v2.3d stably transfected HEK-293 cells. Superimposed traces of two representative recordings from cells preincubated for 20 min in DMSO or in 5 μ M bafilomycin A₁. Mean values of peak currents from cells preincubated for 20 min in DMSO or in 5 μ M bafilomycin A₁. The cells were depolarised to -90 mV and the holding potential was +30 mV. Cells were superfused either with DMSO or with bafilomycin A₁ (0.3 μ M; grey bar) or were preincubated for 20 min in DMSO or in increasing concentrations of bafilomycin A₁ (0.3, 1 and 5 μ M). The peak current is expressed as percent of control. Mean values $\pm$ SEM.

and no difference was found for the input, which was 0.98 ± 0.12 fold for G1 in three experiments. The blot was stripped, and checked for the presence of C-terminus of Ca_v2.3 (Fig. 2B).

Effects of bafilomycin A₁ on Ca_v2.3 Ca²⁺ current

Bafilomycin A₁ was used to investigate the physiological function of the proposed biochemical interaction of the Ca_v2.3 C-terminus with G1 subunit of v-ATPase. Bafilomycin A₁ is a member of the large plecomacrolide family of natural products. It represents a potent and selective inhibitor of v-ATPase. To test its effect on 2C6 cells, bafilomycin A₁ was used along with the external solution for superfusion. In DMSO superfused control cells, mean peak current was 6.0 ± 1.5 pA/pF ($n = 6$ cells), which was similar to cells superfused with bafilomycin A₁ (0.3 μ M) which was 7.9 ± 1.4 pA/pF ($n = 11$ cells; Fig. 3A). And for DMSO preincubated control cells, peak current was 7.2 ± 3.2 pA/pF ($n = 4$ cells), and for bafilomycin A₁ (0.3 μ M) preincubated cells, peak current was 7.9 ± 1.4 pA/pF ($n = 5$ cells; Fig. 3B).

In another set of experiments, the bafilomycin A₁ concentration was increased to 5 μ M. In control cells preincubated with DMSO, peak current was 15.7 ± 6.5 pA/pF ($n = 3$ cells). However, when cells were preincubated with bafilomycin A₁, peak current was reduced significantly ($p = 0.036$) to 3.2 ± 1.6 pA/pF ($n = 6$ cells, Fig. 3C, D). This reduction of current turned out to be irreversible during subsequent washout suggesting that bafilomycin A₁ by inhibiting endogenous v-ATPase may influence Ca_v2.3 mediated current in 2C6 cells.

Effects of bafilomycin A₁ on the NiCl₂-mediated stimulation of the ERG b-wave amplitude

To test the effect of bafilomycin A₁ in a more physiological set-up [27, 30], this drug was applied to an *ex vivo* test system of isolated bovine retina. Retinal signalling was analysed and the electroretinogram of the isolated bovine retina was used to measure retinal activity under scotopic conditions. Adding NiCl₂ (15 μ M) to the superfusing nutrient solution, increases b-wave amplitude 1.3 fold, similar to antagonist SNX-482 [27]. In comparison to SNX-482, the NiCl₂-mediated effect developed faster and was reversible upon washout [30]. Using this protocol before and after adding NiCl₂, we can distinguish the b-wave under basic conditions and after blocking by NiCl₂.

It is well known from the literature that L-type Ca²⁺ channels contribute to retinal signalling. Further, it was found that Ca_v1.2 interact with G2 subunit of v-ATPase [20]. Therefore, we first analysed if bafilomycin may influence basic retinal signalling (Fig. 4A and 4B). At 0.01 % DMSO, no effect was detected on the b-wave amplitude during control conditions, in which we first ap-

Fig. 4. Bafilomycin A₁ reduces the Ni²⁺-induced increase of the ERG b-wave amplitude in the vertebrate retina. Isolated segments of the bovine retina were equilibrated with nutrient solution to establish a stable response during repetitive stimulation with light flashes (every 5 min for 0.5 sec). This equilibration lasted for up to two hours. Subsequently, either the solvent DMSO (0.01%) or bafilomycin A₁ (5 μM) was superfused for 1 hour. Before applying NiCl₂, the solvent carrier or bafilomycin A₁ were washed off during a 20 min period with repetitive light flashes as before. A1. The superfusion of the bovine retina with DMSO (0.01 %) does not change the b-wave amplitude significantly. After washout and subsequent NiCl₂ superfusion, the b-wave amplitude was increased 1.3-fold. During the second washout, the increase was partially reversed. A2. The latency of the b-wave was recorded under the conditions as described in A1. B. Original traces for the ERGs in panel A1 as indicated by roman numbers. C1. No significant increase of the ERG b-wave amplitude by NiCl₂ was observed when the retina was incubated for 1 hour in bafilomycin A₁. C2. Latency times for the recordings of b-wave amplitude in C1. D. Original traces for the ERGs in panel C1 as indicated by roman numbers. E. Mean values from four (controls) to six (bafilomycin) independent experiments for the b-wave amplitude after adding 0.01 % DMSO or ethanol (control), or bafilomycin A₁ (bafilo). Ratios were calculated as b-wave amplitude before and after adding solvent carrier or bafilomycin A₁. F. Mean values for the b-wave amplitude after adding NiCl₂. Ratios were calculated as the b-wave after NiCl₂ application divided by the amplitude after the second washout. Ratios were determined after only using DMSO or after superfusion of bafilomycin A₁ (bafilo).

plied the drug of interest during 1 hour, then, perfused with the washing solution for 35 min. The b-wave ampli-

tude was $15.4 \pm 0.6 \mu V$ (n = 7) before, and $17.0 \pm 0.6 \mu V$ (n = 10) after applying 0.01 % DMSO (p = 0.09;

Experiment (DMSO-/ethanol -control or bafilomycin- experiment)	Column-number:	Amplitude of b-wave, μV				Comparison of ratios	
		Before application	After adding bafilomycin (or solvent only)	After adding bafilomycin (or solvent) plus 15 μM NiCl_2	After Washout	Ratio of column # 3 / # 2	Ratio: column # 3 / # 4
		1	2	3	4	5	6
DMSO-control	mean:	15,43	17,00	25,30	20,67	1,49	1,22
0.01 % (v/v)	SEM (n):	0,57 (7)	0,61 (10)	0,84 (10)	0,67 (3)		
DMSO-control	mean:	15,20	18,60	23,20	18,20	1,25	1,27
0.01 % (v/v)	SEM (n):	0,33 (10)	0,73 (10)	0,33 (10)	0,36 (10)		
Ethanol-control	mean:	11,50	16,75	34,00	23,78	2,03	1,43
0.01 % (v/v)	SEM (n):	1,26 (4)	0,75 (8)	0,63 (5)	0,70 (9)		
DMSO-control	mean:	18,80	16,60	23,71	17,50	1,43	1,35
0.01 % (v/v)	SEM (n):	1,16 (10)	0,43 (10)	0,52 (10)	0,50 (4)		
	mean	15,23	17,24	26,55	20,04	1,55	1,32
	Stadev	2,98	0,92	5,04	2,84	0,34	0,09
	SEM	1,49	0,46	2,52	1,42	0,17	0,05
	n =	4	4	4	4	4	4
Bafilo, 5 μM (in 0.01 % DMSO)	mean:	6,40	6,40	7,90	7,14	1,23	1,11
	SEM (n):	0,31 (10)	0,27 (10)	0,23 (10)	0,26 (7)		
Bafilo, 5 μM (in 0.01 % DMSO)	mean:	20,20	22,20	22,64	19,20	1,02	1,18
	SEM (n):	0,70 (10)	0,63 (10)	0,32 (10)	0,8 (5)		
Bafilo, 5 μM (in 0.01 % DMSO)	mean:	43,60	65,00	76,50	68,00	1,18	1,13
	SEM (n):	2,19 (10)	1,50 (10)	0,71 (10)	0,0 (4)		
Bafilo, 5 μM (in 0.01 % DMSO)	mean:	30,67	37,67	39,15	35,20	1,04	1,11
	SEM (n):	1,12 (6)	0,33 (8)	1,05 (6)	0,49 (5)		
Bafilo, 1 μM (in 0.01 % EtOH)	mean:	16,60	15,20	14,20	15,50	0,93	0,92
	SEM (n):	0,6 (10)	0,44 (10)	0,36 (10)	0,33 (8)		
Bafilo, 1 μM (in 0.01 % EtOH)	mean:	33,55	28,89	29,89	28,00	1,03	1,07
	SEM (n):	0,64 (11)	0,75 (9)	0,63 (10)	0,37 (6)		
	mean	25,17	29,23	31,71	28,84	1,07	1,08
	Stadev	13,35	20,58	24,58	21,52	0,11	0,09
	SEM	5,45	8,40	10,03	8,79	0,05	0,04
	n =	6	6	6	6	6	6

Tab. 1. Effect of bafilomycin on retinal b-wave amplitude. Either the carrier for bafilomycin (0.01 % DMSO or Ethanol; see upper panel) or the bafilomycin itself (1 or 5 μM , lower panel) was added to the isolated and superfused bovine retina. In individual lines, the results from 4 independent control experiments (see upper panel) and 6 bafilomycin experiments (see lower panel) are summarized. For each experiment a fresh bovine retina was used. The columns with data are numbered and contain the mean values from consecutive ERGs (n = number of consecutive ERGs) as the amplitude of the b-wave either after reaching equilibrium ("Before application", column 1), or after adding individual drugs as indicated in column 2 and 3. To minimize the effect of a routinely observed slow run-up, we also calculated the b-wave amplitude after washout in column 4. For each condition (control versus bafilomycin) we could observe in 2 out of three experiments each that a small run-up was present and therefore the ratio of "washout" divided by the amplitude in the presence of NiCl_2 (see column 6) was smaller than the ratios found in column 5 (= amplitudes in column #3 divided by amplitude in column #2).

Fig. 4A1). Finally, we added NiCl_2 , which increased the b-wave amplitude significantly to $25.3 \pm 0.8 \mu\text{V}$ (n = 10; $p < 0.001$). After washout of NiCl_2 , b-wave am-

plitude decreased to $20.7 \pm 0.7 \mu\text{V}$ (n = 3), which was also highly significant compared to the amplitude under NiCl_2 ($p < 0.001$). Similar results were obtained

in two other experiments (Table 1). Not only the increase with bafilomycin, but also the fraction of the washouts in the individual experiments were calculated (Table 1).

The time-to-peak values were also recorded under the different recording conditions. It increased during application of NiCl_2 but not during that of DMSO (Fig. 4A2). This increase in latency is routinely observed after NiCl_2 application [27]. As it is also observed during NiCl_2 application after bafilomycin perfusion, and cannot be easily removed after washout, it may relate to additional unspecific effects of NiCl_2 (Fig. 4C2).

It is important that the NiCl_2 mediated increase has to be reversible (Fig. 4A). The ratio of b-wave amplitude in NiCl_2 divided by the amplitude after the second wash out was 1.28 ± 0.04 ($n = 3$ retinas). This ratio was significantly reduced ($p = 0.03$, Fig. 4F) to 1.14 ± 0.02 ($n = 3$) when the retina was initially superfused with bafilomycin A_1 (Fig. 4C, and 4D). Adding bafilomycin A_1 showed a slight tendency of increase in b-wave amplitude, from $20.2 \pm 0.7 \mu\text{V}$ ($n = 10$) to $22.2 \pm 0.6 \mu\text{V}$ ($n = 10$), which reached the level of significance ($p = 0.047$; Fig. 4E), showing that basal retinal signalling and the activity of $\text{Ca}_v1.2$ were not impaired by bafilomycin A_1 . Rather, the increase by bafilomycin A_1 may be interpreted as an effect on $\text{Ca}_v2.3$ which subsequently reduces the possible attenuation by NiCl_2 . Adding NiCl_2 did not change b-wave amplitude significantly ($22.7 \pm 0.3 \mu\text{V}$, $n = 10$, $p = 0.53$). Therefore, the significant reduction of the NiCl_2 -mediated increase of the ERG b-wave amplitude by bafilomycin A_1 afterwards leads to the suggestion that $\text{Ca}_v2.3$ may interact with the retinal v-ATPase functionally.

Discussion

Construction of the bait

The cytosolic linker between domain II and III, which was selected as a bait in a recent report [9], as well as the C-terminus (this report) were identified as important targets for functional protein interaction partners. Parallel to the detection of the G1 subunit of v-ATPase, also calmodulin-2, a well known interacting protein, was identified by the same Y2H screening procedure (Kamp & Schneider, unpublished results). To ensure that all possible candidates for the $\text{Ca}_v2.3$ C-terminus would be detected, we had selected the full-length C-terminal sequence of the longest $\text{Ca}_v2.3d$ splice variant [21] which contains an exon 45 encoded segment of 43 extra amino acids [31].

Immunoprecipitation of G1-subunit with C-terminus of $\text{Ca}_v2.3$

The FLAG immunoprecipitation in 293T cells confirmed the interaction of the G1 with C-terminus of the channel. This result is in line with a previous report from Y2H that C-terminus of $\text{Ca}_v1.2$ interacts directly with another G-subunit, the G2 of v-ATPase [20].

The finding that an interconnecting subunit between the V0-membrane and V1-peripheral sector of v-ATPase interacted with the C-terminus of the $\text{Ca}_v2.3$ is extremely interesting since V0 was thought to participate in yeast vacuolar fusion [32, 33]. Even more interesting, in *Torpedo*, the V0 subunit $\alpha 1$ was found to colocalize with nerve terminals and to interact with SNARE proteins [34]. Finally, also in *Drosophila* it was shown that this V0 $\alpha 1$ subunit homolog is needed for a late step in synaptic vesicle exocytosis [35]. Therefore, we assumed that the interference of a VGCC with such a protein complex would be functionally important.

Bafilomycin A_1 effects on Ca^{2+} current

Consecutively, bafilomycin A_1 influenced $\text{Ca}_v2.3$ current in 2C6 cells. Bafilomycin A_1 was tested at three different doses, 0.3, 1 and 5 μM . It affects Ca^{2+} currents strongly after preincubation at 5 μM . Therefore, we used 5 μM bafilomycin A_1 for retinal signalling. It is not possible to conclude from the patch-clamp experiments how bafilomycin A_1 may act on $\text{Ca}_v2.3$, because it was thought to block v-ATPase mediated acidification of vesicles mainly [36]. However, one may speculate that the binding and interaction of bafilomycin A_1 with the v-ATPase may cause a feedback modulation on to $\text{Ca}_v2.3$ for which interaction is a prerequisite. Alternatively, the trafficking of $\text{Ca}_v2.3$ to the plasma membrane may be impaired by bafilomycin A_1 , as it was shown for $\text{Ca}_v1.2$ in *X. laevis* oocytes [37], which also may occur in $\text{Ca}_v2.3$ -transfected HEK-293 cells.

While testing retinal signalling, we also took advantage of the observation that bafilomycin A_1 acts irreversibly on 2C6 cells. This was favourable for the recording of ERG b-waves from isolated bovine retina where we could separate bafilomycin A_1 incubation from the consecutive NiCl_2 application.

Bafilomycin A_1 and its effect on the ERG b-wave

The isolated vertebrate retina has proven to be a useful test system for $\text{Ca}_v2.3$ mediated reciprocal signalling, because the selective antagonist of R-type Ca^{2+} current, SNX-482 (30 nM), reveals similar results as the less selective antagonist NiCl_2 (15 μM) [27, 30]. Further, the

retinas from $\text{Ca}_v2.3$ -deficient mice lose partially the NiCl_2 -mediated effects [38], indicating that $\text{Ca}_v2.3$ contributes partially to transretinal signalling.

The solvent of bafilomycin A_1 , DMSO had no effect on retinal signalling at the tested dilution. Bafilomycin A_1 itself did not specifically affect the basal retinal signal transduction but it selectively attenuated the NiCl_2 -mediated increase of the ERG b-wave. This effect was dependent on a preincubation of the retina in bafilomycin A_1 leading to the suggestion that (i) either an impairment of the vesicle acidification or (ii) a disturbed formation of a fusion pore could account for this attenuation, or both. It is not known for $\text{Ca}_v1.4$ that it interacts with any of the G-subunits of v-ATPase but such an interaction is well accepted for $\text{Ca}_v1.2$ which binds to the G2 subunit of v-ATPase. As bafilomycin A_1 does not interfere with the basal retinal signal transduction before applying NiCl_2 , one must conclude that neither this L-type channel mediated signal transduction nor the acidification of vesicles in general is impaired by bafilomycin A_1 during the fast recorded retinal signalling. Finally, the reported bafilomycin A_1 effect on the NiCl_2 mediated, R-type Ca^{2+} channel dependent retinal signalling may point to a new role of $\text{Ca}_v2.3$, triggering fusion pore formation via G1 of v-ATPase during exocytosis and neurotransmitter release.

Implications for exocytosis

It is widely accepted that VGCC form an integral part of the exocytotic machinery [39–41]. New results indicate that the V0-domain of the v-ATPase also plays an important role during exocytosis and perhaps may act as fusion pore [32, 34, 35, 42]. During exocytosis the participating proteins associate and form protein complexes, called excytosome [43] or synaptosecretosome [44]. Such a view of a proteinous fusion pore is still under discussion. Most recently additional evidence was found for such a model of a molecular link between the v-ATPase and the SNARE-mediated fusion, because a structural and functional interaction between V0 c-subunit and the v-SNARE synaptobrevin was reported [45, 46].

Thus, the interaction between $\text{Ca}_v2.3$ and the G-subunit may be important for such a formation of protein complexes. The binding of the $\text{Ca}_v2.3$ C-terminus to the “stalk” subunit G1 may be necessary to separate the V1 from the V0 complex. G-subunits of v-ATPase have been shown to be crucial for controlling the stability of the yeast v-ATPase holoenzyme [47]. Therefore, we assume that after acidification of the synaptic vesicles and their filling with neurotransmitters, the v-ATPase holoenzyme may be destabilized and consecutively may dissociate into sepa-

rate V1- and V0 domains, through G1 interaction with $\text{Ca}_v2.3$. The V0-subunit or parts of it may now be prepared for forming and opening of the fusion pore in close connection to a calcium channel, as postulated during vacuole membrane fusion [48].

Because Ca^{2+} as the final trigger for the fusion enters the cell also through $\text{Ca}_v2.3$, such a close association of a Ca^{2+} channel with the interconnecting subunit may act as an important switch for secreting neurotransmitters. This interpretation may also explain our retina results, where preferably the release of inhibitory neurotransmitters may be affected. In such a sense, the L-type channel triggered glutamate release must act in a bafilomycin independent way, either by docking to other subunits of the v-ATPase or by docking to another protein for forming the fusion pore, or forming no fusion pore at all but following the full collapse mode as a more classical way of neurotransmitter release [49].

Nano-environments of Ca_v2 Ca^{2+} channels have been investigated recently by quantitative proteomics [50]. Interestingly, $\text{Ca}_v2.3$ (and $\text{Ca}_v2.2$) was proven to interact with subunits B and H of the V_1 sector of the v-ATPase complex, in which the G1 subunit is also incorporated. Most recently, R-type Ca^{2+} channels were proven to control Ca^{2+} microdomains at parallel fiber to Purkinje cell synapses during the induction of presynaptic long-term potentiation (LTP) [51], indicating that biochemical determination of protein-protein interactions will be helpful to understand how R-type channels might control the vesicular pool and not only the Ca^{2+} triggered neurotransmitter release.

For $\text{Ca}_v2.3$ it was shown that it participates in LTP at the mossy fiber/CA3 synapse [52, 53]. Further, other regions in the brain, which reveal presynaptic LTP [54], are also known to express $\text{Ca}_v2.3$. Future studies may elucidate how such a mechanism may work, which includes $\text{Ca}_v2.3$ -mediated Ca^{2+} regulation of presynaptic processes other than neurotransmitter release.

Implications for Ca^{2+} triggered regulations

For C-terminus of VGCC, several interacting proteins have been identified. Some of them are connected to a Ca^{2+} mediated feedback on the ion channel itself including facilitation and inhibition. Calmodulin, Ca^{2+} binding proteins [55], and EFHC1 [19] are involved in important regulations during cellular homeostasis and development [15]. G1 of v-ATPase may extend the function of the C-terminus to an important process as the secretion and therein the formation of a fusion pore itself. Future studies are required to identify the full set of protein which

may help to scaffold the secretion process, especially the fast “kiss-and-run” mode of neurotransmitter secretion at elevated Ca^{2+} concentrations [56, 57]. Such an incomplete incorporation of the vesicle membrane may really depend on the existence of a fusion pore. Finally, any kind of an interaction of $\text{Ca}_v2.3$ with the v-ATPase may shed some light on the mechanism of $\text{Ca}_v2.3$ dependent regulation of apoptosis during the development of juvenile myoclonic epilepsy [19] or during experimentally induced epilepsy in mice [58], especially because Hsp70, which interacts with the II-III loop, is involved in the uncoating of clathrin from coated vesicles. A well regulated endocytosis and connected uncoating is needed for a regulated degradation of membrane material during apoptosis [59].

Abbreviations

v-ATPase (Vacuolar-type H^+ -translocating ATPase); SNARE (Soluble N-ethylmaleimide-sensitive factor attachment protein receptor); VGCC (Voltage Gated

Calcium Channels); Y2H (Yeast two Hybrid); C-terminus (Carboxyl terminus).

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