The Molecular Chaperone hsp70 Interacts with the Cytosolic II-III Loop of the Ca_v2.3 E-type Voltage-gated Ca²⁺ Channel

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Key Words

Protein interaction • 15-deoxyspergualin • Toxin-resistant current • R-type • Secretion • Protein kinase C • Facilitation

Abstract

Multiple types of voltage-activated Ca²⁺ channels (T, L, N, P, Q, R type) coexist in excitable cells and participate in synaptic differentiation, secretion, transmitter release, and neuronal plasticity. Ca²⁺ ions entering cells trigger these events through their interaction with the ion channel itself or through Ca²⁺ binding to target proteins initiating signalling cascades at cytosolic loops of the ion conducting subunit (Ca_vα1). These loops interact with target proteins in a Ca²⁺-dependent or independent manner. In Ca_v2.3-containing channels the cytosolic linker between domains II and III confers a novel Ca²⁺ sensitivity to E-type Ca²⁺ channels including phorbol ester sensitive signalling via protein kinase C (PKC) in Ca_v2.3 transfected HEK-293 cells. To understand

Ca²⁺ and phorbol ester mediated activation of Ca_v2.3 Ca²⁺ channels, protein interaction partners of the II-III loop were identified. FLAG-tagged II-III - loop of human Ca_v2.3 was over-expressed in HEK 293 cells, and the molecular chaperone hsp70, which is known to interact with PKC, was identified as a novel functional interaction partner. Immunopurified II-III loop-protein of neuronal and endocrine Ca_v2.3 splice variants stimulate autophosphorylation of PKCα, leading to the suggestion that hsp70 - binding to the II-III loop - may act as an adaptor for Ca²⁺ dependent targeting of PKC to E-type Ca²⁺ channels.

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Introduction

Structure and function of voltage-gated Ca²⁺ channels have been analyzed in great detail during the last two decades [1, 2]. This paves way for the molecular dissection of related inherited diseases called

channelopathies [3-7]. Detecting interacting proteins in large scale even extends the molecular view to large networks of proteins which are important for normal function and may be involved in several disease states [8].

The ion conducting $\text{Ca}_v\alpha 1$ subunits of high-voltage activated non-L-type Ca^{2+} channels share a high degree of structural similarity within the conserved transmembrane domains. But their interconnecting II-III loops are structurally highly divergent. Within this region, splice variants of $\text{Ca}_v 2.3$ differ by an arginine-rich segment which is conferring a unique Ca^{2+} -sensitivity to the major neuronal $\text{Ca}_v 2.3$ splice variants [9-11]. The increasing cytosolic Ca^{2+} concentration serves as a positive feedback signal for $\text{Ca}_v 2.3$ E-type Ca^{2+} channels. We assumed that Ca^{2+} -sensitive proteins might interact with the II-III – loop of $\text{Ca}_v 2.3$, because a direct binding of cytosolic Ca^{2+} to the positively charged arginine-rich segment is very unlikely. As the novel Ca^{2+} -sensitivity of neuronal $\text{Ca}_v 2.3$ subunits was detected in the $\text{Ca}_v 2.3$ transfected human embryonic kidney cell line (HEK-293), the same cell system was used to identify interaction partners by immunoprecipitation of the over-expressed FLAG-tagged II-III loop. This approach led to the detection of hsp70 as a novel interaction partner which was functionally tested in $\text{Ca}_v 2.3$ stably transfected HEK-293 cells and during *ex vivo* retinal signal transduction. Further, it was found that the immunopurified II-III loop of $\text{Ca}_v 2.3$ stimulates autophosphorylation of protein kinase C.

It will be discussed that the interaction complex identified so far may involve additional scaffold proteins providing specificity for the observed phorbol ester effects on extended neuronal $\text{Ca}_v 2.3c$ and $\text{Ca}_v 2.3d$ splice variants containing the exon 19 encoded arginine-rich segment within the II-III loop [9-11].

Materials and Methods

Materials

15-Deoxyspergualin was from Nippon Kayaku (Tokyo, Japan). L- α -Phosphatidyl-L-Serine, phorbol 12-myristate 13-acetate, the FLAG tagged immunoprecipitation kit 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 for western blots 1:5000 as recommended by the company. PKC α was purchased as purified protein from Calbiochem (Merck Biosciences, Schwalbach, Germany), and was over-expressed in HEK-293 cells using the pPKC α -EGFP vector from Clontech (Heidelberg, Germany). The bicistronic mammalian expression vector, pIRES-hrGFP-1a, was purchased from Stratagene

(Amsterdam, The Netherlands), and the vector providing an in-frame myc epitope, pcDNA3.1/myc-His, was from Invitrogen (Karlsruhe, Germany). The restriction enzymes were purchased from Fermentas GmbH (St. Leon-Rot, Germany).

DNA-vectors

The full length cDNA of $\text{Ca}_v 2.3$ was cloned from fetal brain [12]. It corresponds to the splice variant $\text{Ca}_v 2.3d$ (α_{1Ed} , GenBank # L27745) which was subcloned into the pcDNA3 vector for transient or stable expression in HEK-293 cells [13]. The deletions of amino acids in $\text{Ca}_v 2.3d$ to generate $\text{Ca}_v 2.3e$ and related mutants were made by a double step overlap extension PCR [14]. The splice variant $\text{Ca}_v 2.3e$ detected in INS-1 cells [15] lacks exon 19 (nts 2242 - 2298, 19 aas) of $\text{Ca}_v 2.3d$. The mutant $\text{Ca}_v 2.3d\text{RDEL}$ lacks exon 19 and the adjacent 19 amino acid residues homologous to exon 19 (nts 2242 - 2355) while a second mutant, $\text{Ca}_v 2.3d\Delta R_{2299-2355}$, lacks only the last 19 aas encoded by nts 2299 - 2355 (see Fig. 1).

The full length II-III – loop construct was amplified from the $\text{Ca}_v 2.3d$ -pcDNA3 vector [13] with forward primer containing the Not I site and Kozak sequence (5'-ATGCGGCCGCCACCACCATGGATAATCTCGCCAACGCCAG-3'), and the reverse primer containing a Sal I site (5'-ACGTCGACGGCGCAGGTTACACGAT-3'). It was digested with Not I and Sal I, and subcloned into pIRES-hrGFP-1a yielding pIRES-hrGFP-1a- $\alpha 1E$ -II-III loop. Further, the Not I – Sal I fragment was also subcloned into pBluescript-SK to be used as a template to generate shorter II-III – loop variants. The Pst I – Bpu1102 I fragments from pBSK-f $\alpha 1E\Delta$ ins1 [9;10], from pBSK-f $\alpha 1E$ -1/2Rdel and from pBSK-f $\alpha 1E$ -Rdel [9, 10] were subcloned into the Pst I – Bpu1102 I digested pBSK- $\alpha 1E$ -II-III-loop vector. The Not I – Sal I fragments obtained from last three intermediate vectors were subcloned into the Not I – Sal I digested pIRES-hrGFP-1a vector yielding the final constructs used for transfection into HEK-293 cells, the vectors pIRES-hrGFP-1a- $\alpha 1E$ -II-III-loop- Δ Ex19 (= $\text{Ca}_v 2.3e$), pIRES-hrGFP-1a- $\alpha 1E$ -II-III-loop- Δ 1/2Rdel (= $\Delta R20$), and pIRES-hrGFP-1a- $\alpha 1E$ -II-III-loop- Δ Rdel (= $\Delta R19\Delta R20$), respectively.

The carboxyl terminus of each II-III loop-segment was fused to three FLAG-epitopes, (N-Asp-Tyr-Lys-Asp-Asp-Asp-Lys-C)₃, for immunoprecipitation [16, 17].

Clones containing A2B56 phosphatase and hsp70 were purchased from the Research Center Berlin (Deutsches Ressourcenzentrum für Genomforschung, GmbH). The coding sequence for hsp70 was amplified from clone IRALp96200740 using Hind III site containing forward primer, 5'-CCC AAG CTT CCC GCC GCC ATG GCC AAA GCC GCG GCG ATC-3' and Xho I site containing reverse primer, 5'-AAC TCG AGC CAT CCA CCT CCT CAA TGG TAG G-3'. The cDNA-fragment was digested with Hind III and Xho I, and subcloned into pcDNA3.1/myc-His. PKC α was amplified from a commercially available vector (Clontech, Heidelberg, Germany) and subcloned in the same way. The constructs were sequenced using standard methods.

Transfection of HEK-293 cells

For voltage clamp recordings, HEK-293 cells were transfected with the vectors of interest and a vector expressing

the enhanced green fluorescent protein (EGFP) using the calcium-phosphate precipitation technique (Stratagene, Amsterdam, The Netherlands). For the patch-clamp studies, all variants of Ca_v2.3- α 1 subunits were expressed in the pcDNA3 vector.

For immunoprecipitation, HEK-293 cells were transfected with different vector constructs containing variants of the II-III – loop from Ca_v2.3 and pIRES-hrGFP-1a vector backbone was used as a control (see Fig. 1B and: [9, 10]).

Immunoprecipitation of the FLAG-tagged protein and phosphorylation

The II-III – loop was cloned into the pIRES-hrGFP-1a vector in frame with 3 consecutive FLAG-tags. The transfected HEK 293 cells were lysed using a buffer consisting of (mM): Tris HCl, 50 (pH 7.4); NaCl, 150; EDTA, 1; Triton X-100, 1 % (v/v). The FLAG-tagged proteins were isolated by affinity chromatography using anti-FLAG agarose matrix, according to the instructions of the manufacturer (product number FLAGIPT-1, Sigma, Munich). All steps were performed at 4°C. The bound FLAG-fusion proteins were eluted either with the denaturing sample buffer for interaction studies, or with the 3X FLAG peptide solution (product number F4799, 150 ng/ μ l final peptide concentration) for the phosphorylation assays.

Tagged proteins were phosphorylated *in vitro* by PKC α (Calbiochem) using gamma ³²P-ATP [16]. The reaction was started by addition of 2 nM PKC α and stopped after 30 min by addition of denaturing sample buffer. Incorporated ³²P was analyzed after SDS/PAGE by autoradiography.

Western blotting

HEK-293 cells were grown as described [14]. Proteins were separated by polyacrylamide gel electrophoresis according to standard protocols and were immunoblotted as reported previously [14]. The ECL detection kit was purchased from Amersham Corp. (Braunschweig, Germany).

Mass spectrometry

Reflectron MALDI-TOF mass spectrometry was performed in the central service of the Center for Molecular Medicine (Köln), on a Bruker Reflex III or IV instrument (Bruker Daltonik, Bremen, Germany) in the positive ion mode using a pulsed laser beam (nitrogen laser, λ = 337 nm). The acceleration voltage was set to 20 kV, and the reflector voltage was 22.5 kV.

Voltage-clamp recordings

Membrane currents from transfected HEK-293 cells were recorded in the tight-seal whole-cell configuration of the patch clamp technique [10, 11] using either 5 mM Ca²⁺ or 15 mM Ba²⁺ as charge carrier in the external solution as indicated. Ca_v2.3 transfected HEK-293 (HEK-2C6) cells were voltage-clamped at -90 mV using the EPC-9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). The cell capacitance varied between 12 pF to 57 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). The resistance of the pipette tip was in the

range of 2 M Ω to 5 M Ω 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, pH 7.35 with HCl (osmolality: 305 - 320 mosmol/kg). All experiments were performed at room temperature (22-24°C). Gravitation 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). 15-deoxyspergualin was dissolved in double distilled water and prepared as a 1000-fold stock solution.

Data analysis and curve fitting

Current amplitudes and kinetics were recorded as reported [10]. Inactivation kinetics of the currents were estimated by fitting the decaying part of the current traces with the equation: $I(t) = a_0 + a_1 \cdot \exp(-(t-x)/\tau_1)$. 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.

ERG recordings from the isolated superfused bovine retina [18]

Bovine retina was isolated and perfused with a nutrient solution, which either contained the drug of interest or lactose the excipient for control [19, 20].

Eyes were removed from a calf in the local slaughterhouse and immediately stored in an empirically optimized nutrient solution consisting of (in mM): 120 NaCl, 2.0 KCl, 0.15 CaCl₂, 0.1 MgCl₂, 1.5 NaH₂PO₄, 13.5 Na₂HPO₄, and 5 glucose with a final pH of 7.8. Eyes were hemisected at the junction of sclera and cornea followed by removal of lens and vitreous body from the posterior eyecup. Using a 7 mm trephine, circular pieces were obtained from the posterior segment, which had been divided into four sclera-choroid-retina segments prior to extraction. Subsequently, the retina was detached from the underlying pigment epithelium by gentle shaking. Plain retina segments from calf were then transferred to a chamber sandwiched between a mesh and an O-ring, and finally transferred to a perfusion chamber located in an electrically and optically insulated box [18]. The whole preparation was carried out under dim red illumination. Prior to experiments the retina was continuously superfused on both sides using flow system fed from a reservoir 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 at intervals of 5 min. The flash intensity at the retinal surface was set to 6.3 mlx using calibrated neutral density filters. Light stimulation lasting for 500 ms was controlled by a timer operating mechanical shutter system. To avoid 50 Hz noise, the heater was shut down during light stimulation and ERG recording. 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.

NiCl₂ was added from a 10 mM stock solution. Drug

Fig. 1. Splice variants of $\text{Ca}_v2.3$ and vector for over-expression in HEK-293 cells. A.) Major differences between $\text{Ca}_v2.3$ splice variants reside in the II-III – loop (exon 19) and the carboxyl terminus (exon 45). The $\text{Ca}_v2.3d$ variant was cloned from human fetal brain cDNA library [12], and the $\text{Ca}_v2.3c$ variant is the major $\text{Ca}_v2.3$ transcript found in cerebrum while the $\text{Ca}_v2.3e$ variant is expressed in cerebellum, heart and in endocrine tissues [14, 15]. Note, that the splice variants expressing exon 19 (insert R19) were shown to be modulated by cytosolic Ca^{2+} [9;10]. Note, exon 19 encodes the full R19 insert but R20 is only encoded by the first 57 nts of exon 20. B.) Vector constructs for the over-expression of FLAG-tagged II-III loop segments from $\text{Ca}_v2.3$. B1) Cartoon of the pIRES-hrGFP-1a vector showing the insertion positions. B2) Dimensions of the individual segments from the II-III loop which were fused in frame to a triple FLAG-epitope for immunoprecipitation.

application was initiated after the b-wave had reached a 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.

Results

Search for protein binding partners of cytosolic II-III loops from $\text{Ca}_v2.3$ splice variants

To understand the Ca^{2+} and phorbol ester mediated stimulation of $\text{Ca}_v2.3$ E-type Ca^{2+} channels, different II-III loops of $\text{Ca}_v2.3$ splice variants, either containing or lacking the exon 19 encoded arginine rich segment (R19), were over-expressed in HEK-293 cells. Further, two mutants were generated (Fig. 1). The immunopurified proteins were resolved on a polyacrylamide gel (Fig. 2). Two polypeptides larger than 100 kDa, and another one with 45 kDa were present in all aliquots loaded on the gels including aliquots from the untreated beads (Fig. 2, lane 2 to 5) suggesting that these bands represent unrelated material. From HEK-293 cells transfected with the full length II-III loop, polypeptides of 79, 74, and 72 kDa were precipitated (Fig. 2, lane 3) and clearly stained by Coomassie blue as major proteins. They were co-immunoprecipitated with the 66 kDa II-III – loop fragment

(see lower arrow, Fig. 2) which was identified by using anti-FLAG antibodies (not shown). Based on the primary sequence of the full length II-III loop (1347 nts) and the fused segments, a molecular weight of 54 kDa was calculated which is lower than the apparent size of the FLAG-tagged full length II-III – loop (66 kDa). This maybe due to glycosylation or electrostatic interactions of the highly charged FLAG-tags with the polyacrylamide matrix.

In parallel, HEK-293 cells were transfected with the shorter variant of the II-III loop, lacking R19 (Fig. 2, lane 6). From mock transfected cells (Fig. 2, lane 2), a 72 kDa polypeptide observed in lane 3 to 6 was also co-immunoprecipitated leading to the conclusion, that this one is unrelated and that the 79 kDa sized polypeptide from the full length loop of $\text{Ca}_v2.3d$ (Fig. 2, upper arrow, lane 3) may represent a specific interaction partner for the co-immunoprecipitated II-III loop of neuronal $\text{Ca}_v2.3$ splice variants. As the precipitated 79 kDa polypeptide resembles the size of PKC α and because the 79 kDa protein was easily detected by Coomassie blue staining, its band was excised and trypsin digested for mass spectrometry. Proteins were identified from the resulting mass spectra using the commercially available search engine Mascot® (Matrix Sciences, London, UK). Two

Fig. 2. The full length FLAG-tagged II-III – loop specifically co-immunoprecipitates with a 79 kDa polypeptide. Four different II-III – loop constructs (Fig. 1) were overexpressed in HEK-293 cells. The crude extract was immunopurified through columns and the eluate was loaded on a 5 – 20 % polyacrylamide gradient gel. The proteins are detected after Coomassie blue staining. Size markers are loaded on lane 1 and 8. The eluate from mock transfected HEK-293 cells (lane 2) and the eluate from untreated beads (lane 7) contain unspecific polypeptide bands. The sizes of the tagged II-III – loop constructs vary between 65,6 kDa (lane 3), 57,6 kDa (lane 4), and 61,1 kDa (lane 5 and 6). The next polypeptide of 71,8 kDa is also precipitated from mock transfected cells. The slightly larger polypeptide of 74,7 kDa is weakly precipitated with full length loop, and not found in II-III- Δ RDEL but clearly observed in both II-III – loop variants which still contain at least one arginine-rich segment (lane 5 and 6). The unique band of the full length II-III – loop has a retention time of a 78.6 kDa polypeptide (lane 3, upper arrow at the left side) and contains the heat shock proteins (Tab. 1). Thus, neuronal and endocrine $\text{Ca}_v2.3$ variants coimmunoprecipitate different interaction partners.

polypeptide-bands were identified by the matrix-assisted laser desorption/ionization – time of flight – mass spectrometry (MALDI-TOF-MS). None of the two peptides represented any PKC fragment. The 66 kDa protein was identical with the human II-III – loop of $\text{Ca}_v2.3$, and the 79 kDa polypeptide was indicative of the human heat shock 70 kDa protein 1A (hsp70) (Tab. 1). From this peptide, the masses of 29 tryptic peptides matched with published hsp70 sequences leading to a total score of 133. In conclusion, hsp70 was detected as a novel interaction partner of $\text{Ca}_v2.3$ II-III loop, but not the prime candidate PKC which is known to activate $\text{Ca}_v2.3$ induced currents, and which may be responsible for the Ca^{2+} dependent effects on inactivation and recovery from inactivation [10, 11].

Both II-III loops of neuronal and endocrine/cardiac $\text{Ca}_v2.3$ co-immunoprecipitate with hsp70.

To confirm the direct binding of hsp70 to $\text{Ca}_v2.3$ -II-III loop, we used another independent approach. PKC α and hsp70 were subcloned into a vector containing a C-terminal myc - tag. These vectors were co-transfected in HEK-293 cells either with one of the two different FLAG-tagged II-III – loop constructs or with the cytosolic carboxyl terminal domain of $\text{Ca}_v2.3$ (corresponding to nts 5169 – 6936, GenBank L27745). After 72 hours, the transfected cells were lysed, and the II-III – loop protein partners were immunoprecipitated and analyzed by SDS-PAGE (Fig. 3). For PKC α (Fig. 3, lane 4-6), non-specific proteins were detected larger than 100 kDa, as with the

Fig. 3. Co-immunoprecipitation of FLAG-tagged II-III loop and c-myc-tagged proteins. After over-expression of different II-III loops, soluble proteins from HEK-293 cells were extracted and immunoprecipitated by anti-FLAG - linked beads. The proteins were eluted by an excess of the FLAG-peptide and were separated by SDS-PAGE. After western blotting, anti - c-myc was used as the primary antibody. Three different partners were expressed in HEK-293 cells, the B56 regulatory subunit of the protein phosphatase 2A (PP2A) as a control (lane 1 to 3), the PKC α (lane 4 to 6) and the hsp70 protein (lane 7 to 9). These proteins were probed by the co-transfection of a mock vector (lane 1, 4, and 7), the vector containing the full length II-III loop from $\text{Ca}_v2.3d$ (lane 2, 5, and 8), and the vector containing the II-III loop from $\text{Ca}_v2.3e$ which is lacking exon 19 encoded R19 (lane 3, 6, and 9). This figure shows the results from one out of three experiments.

Tab. 1. MALDI-TOF analysis of the two major polypeptides separated by PAGE. Both identified proteins show a slower electrophoretic mobility than predicted by their primary sequences which might be explained by electrophoretic interactions caused by the FLAG-tag of the II-III – loop segment.

Polypeptide	Identity	GenBank accession number	Matched mass values / searched mass values	Percent of sequence covered
79 kDa	heat shock 70kD protein 1A [Homo sapiens]	NM_005345	29 / 220	58
66 kDa (predicted size, 53,6 kDa)	voltage-gated Ca^{2+} channel $\text{Ca}_v2.3$ [Homo sapiens]	L27745	25 / 106	42

other putative partners (Fig. 3, lanes 1-3 and 7-9). Also the polypeptide of 90 kDa in the PKC α -experiments (Fig. 3, lane 5,6) must be regarded as non-specific, because it was co-immunoprecipitated with the mock vector, too (Fig. 3, lane 4). Therefore, no specific protein of the expected size was precipitated with the full length II-III loop co-transfected with PKC α or the control protein phosphatase-subunit PP2A. However, the c-myc-tagged hsp70 was co-immunoprecipitated with both II-III loops (Fig. 3, lane 8 and 9), but not with the over-expressed carboxyl terminal domain of $\text{Ca}_v2.3$ (not shown) leading to the conclusion that hsp70 but not PKC α - is indeed a direct interaction partner of $\text{Ca}_v2.3$. Further, hsp70 co-immunoprecipitates with the II-III loops of both $\text{Ca}_v2.3$ splice variants (Fig. 3, lane 7 and 8) although such a 70 kDa polypeptide was not observed in large amounts in the Coomassie-stained gel (Fig. 2), suggesting, that the affinity of the two different $\text{Ca}_v2.3$ II-III loops to hsp70 might differ.

Effect of 15-deoxyspergualin (DSG) on $\text{Ca}_v2.3$ mediated calcium currents

DSG is a synthetic analogue of spergualin, which was originally isolated from the culture filtrate of *Bacillus laterosporus* [22]. It inhibits the nuclear translocation of activated NF-kappaB through heat shock proteins, and reduces the cytoplasmic expression of hsp70 [23]. Assuming that DSG may interact with hsp70, DSG (5 μM) was applied to the external bath solution during patch clamp recordings of Ca^{2+} inward currents from $\text{Ca}_v2.3$ d transfected HEK-293 cells. At 5 μM , DSG significantly reduced the current density from 13.9 ± 1.7 pA/pF to 8.3 ± 1.4 pA/pF ($n = 11$ cells, $p = 0.03$), a reduction that turned out to be irreversible during subsequent washout suggesting that DSG restrains hsp70 from Ca^{2+} channel. Under control conditions with equivalent amounts of lactose, an excipient for DSG, peak I_{Ca} remained unchanged at 13.5 ± 1.6 pA/pF and 12.6 ± 1.1 pA/pF, respectively ($n = 5$ cells, $p = 0.6$).

From previous experiments in $\text{Ca}_v2.3$ -transfected HEK-293 cells, it was known that incoming Ca^{2+} reduces the inactivation of the channel when switching the charge carrier from Ba^{2+} to Ca^{2+} [10]. Therefore we calculated fast and slow inactivation components in 5 mM Ca^{2+} . The decaying parts of the Ca^{2+} current traces were approximated using an exponential fit [9-11] (Fig. 4A). The time constants were compared before and after application of drugs (Fig. 4B and 4C). In lactose, the fast inactivation time constant (τ_{fast}) was increased from 43 ± 5 ms to 56 ± 6 ms ($n = 5$), whereas in DSG, τ_{fast} was decreased from 30 ± 3 ms to 27 ± 3 ms ($n = 11$). The τ_{fast} values between lactose and DSG treatments was significantly different ($p = 0.0001$). Similarly the slow inactivation time constant (τ_{slow}) was slightly increased from 118 ± 6 ms to 128 ± 8 ms ($n = 5$) in lactose, but in DSG, τ_{slow} was significantly decreased from 104 ± 4 ms to 89 ± 8 ms ($n = 9$). The τ_{slow} value in DSG treatment was reduced significantly ($p = 0.008$) from that of lactose treatment. Thus, DSG reduced both fast and slow inactivation of the channel (Fig. 4B and 4C). This observation leads to the conclusion that DSG interferes with the functional binding of hsp70 to the $\text{Ca}_v2.3$ E-type Ca^{2+} channel.

Effect of 15-deoxyspergualin on retinal signal transduction and the Ni^{2+} -sensitive stimulation of the ERG b-wave amplitude

To demonstrate the effect of DSG on II-III loop in a more physiological process, we used the isolated bovine retina as an *ex vivo* test system for neural transmission [18, 19]. It was selected, because the ERG-b-wave from the bovine retina contains a Ni^{2+} -sensitive component which is related to $\text{Ca}_v2.3$ / E-type voltage-gated Ca^{2+} channels [20].

In control recordings lactose, the excipient for DSG was used (Fig. 5A). During repetitive light stimulation (every 5 min for 0.5 sec), lactose by itself caused a 1.2-fold increase of the b-wave response (Fig. 5C). Under

Fig. 4. 15-deoxyspergualine (DSG) accelerates the inactivation kinetics of $Ca_v2.3d$ induced Ca^{2+} inward currents. DSG, when applied to the extracellular bath solution, influences the I_{Ca} carried by $Ca_v2.3d$ in stably transfected in HEK-293 cells (HEK-2C6 cells). Charge carrier concentration was 5 mM. Currents were evoked by depolarisations from -90 mV to 30 mV for 400 ms in the presence or absence of 5 μ M DSG. Lactose, the excipient for DSG, is used as control. A.) Representative current traces were recorded 3.5 min (40 sweeps) after adding either lactose (panel A1) or 5 μ M DSG (panel A2). Both current traces are superposed in panel A3. Note, that the current trace in the presence of DSG is labelled (*). B.) Mean of fast inactivation time constants (τ_{fast}). The τ_{fast} -values were determined before (hatched boxes) and 1 min after adding (black boxes) either lactose as control (lac, $n = 5$) or 5 μ M DSG (dsg, $n = 11$). Decrease with DSG when compared with lactose is significant ($p = 0.0001$). C.) Mean of slow inactivation time constants (τ_{slow}). The τ_{slow} -values were determined before (hatched boxes) and 1 min after adding (black boxes) either lactose (lac, $n = 5$) or 5 μ M DSG (dsg, $n = 9$). In DSG, the difference of the τ_{slow} -values is highly significant ($p = 0.008$).

20 μ M DSG, the b-wave amplitude was unchanged in 4 independent experiments (Tab. 2). Further, the b-wave amplitude was increased from $19.6 \pm 5.7 \mu$ V in lactose to $25.4 \pm 7.3 \mu$ V in 15 μ M $NiCl_2$ plus lactose. After washout of $NiCl_2$ and lactose, b-wave amplitude was completely reduced to $20.1 \pm 5.8 \mu$ V. Such a complete reversal of the b-wave amplitude after washout is indicative for the E-type Ca^{2+} channel and is similar to the recently reported values for a $NiCl_2$ mediated stimulation [20]. We have deliberately used, as the most reliable measure of the Ni^{2+} -effect, its washout by the basic nutrient solution (Fig. 5D) because lactose by itself caused a 1.2-fold increase in b-wave amplitude before $NiCl_2$ application (Fig. 5A).

After equilibrating the retina with 20 μ M DSG, the $NiCl_2$ -mediated maximum response of $29.4 \pm 5.9 \mu$ V was only partially washed out to $27.1 \pm 4.9 \mu$ V ($n = 6$)

(Fig. 5B). In comparison, the $NiCl_2$ -mediated increase of b-wave amplitude was significantly reduced ($p = 0.006$) from 1.27 fold under control conditions to 1.07-fold after DSG application (Tab. 1 and Fig. 5D). As the Ni^{2+} mediated increase was significantly higher under control recordings and completely reversible during washout (Fig. 5A), the reduced ratio after DSG application indicates that a heat shock protein, probably hsp70, is involved in retinal signal transduction. DSG influences both, the basic retinal signalling and more intensely $NiCl_2$ -stimulated propagation of excitation; leading to the conclusion, that hsp70 may influence the b-wave amplitude through its interaction with $Ca_v2.3$. We hypothesize that hsp70 may stabilize the II-III loop or may act as a scaffold protein for protein kinase $C\alpha$ ($PKC\alpha$) favouring $Ca_v2.3$ phosphorylation.

Tab. 2. Effect of 15-deoxyspergualin on retinal b-wave amplitude.

Experiment (Control or DSG)		Amplitude of b-wave, μ V				Ratio: # 3 / # 4
		Before application	After adding 20 μ M DSG (or lactose, only)	After adding 20 μ M DSG plus 10 μ M NiCl ₂	After washout	
		1	2	3	4	5
Control, lactose	mean:	24,0	33,7	42,7	34,0	1,26
	SEM (n):	0,52 (6)	0,95 (6)	0,42 (6)	0,52 (6)	
Control, lactose	mean:	9,3	10,9	14,5	12,4	1,17
	SEM (n):	0,42 (6)	0,76 (6)	0,43 (10)	0,48 (7)	
Control, lactose	mean:	20,2	24,0	32,3	25,2	1,28
	SEM (n):	0,87 (10)	0,73 (10)	0,70 (8)	0,53 (10)	
Control, lactose	mean:	9,1	9,6	12,2	8,9	1,37
	SEM (n):	0,4 (7)	0,6 (7)	0,55 (10)	0,4 (7)	
Mean		15,65	19,55	25,43	20,13	1,27
SD		7,61	11,46	14,61	11,60	0,08
SEM		3,80	5,73	7,30	5,80	0,04
n =		4	4	4	4	4
DSG 20 μ M	mean:	49,3	47,2	53,4	47	1,14
	SEM (n):	0,70 (14)	0,98 (9)	0,74 (7)	0,96 (11)	
DSG 20 μ M	mean:	28,2	25,8	30,4	26,0	1,17
	SEM (n):	0,76 (10)	1,67 (8)	0,56 (9)	0,88 (9)	
DSG 20 μ M	mean:	27,4	27,6	38,0	34,4	1,10
	SEM (n):	0,60 (10)	0,93 (9)	0,58 (9)	0,40 (10)	
DSG 20 μ M	mean:	15,2	11,3	16,5	15,7	1,05
	SEM (n):	0,33 (10)	0,65 (8)	0,50 (8)	0,52 (7)	
DSG 20 μ M	mean:	14,8	15,0	16,5	16,2	1,02
	SEM (n):	0,53 (10)	0,33 (10)	0,33 (8)	0,36 (10)	
DSG 20 μ M	mean:	13,0	18,3	21,7	23,0	0,94
	SEM (n):	0,45 (6)	0,95 (6)	0,85 (6)	0,68 (6)	
Mean		24,65	24,20	29,42	27,05	1,07
SD		13,78	12,88	14,44	11,97	0,08
SEM		5,63	5,26	5,90	4,89	0,03
n =		6	6	6	6	6

Splice variants of Ca_v2.3 are differently affected by phorbol esters although both variants bind hsp70

Ca²⁺ and phorbol esters stimulate splice variants of Ca_v2.3 via the II-III loop differently [10, 11]. E-type Ca²⁺ channels expressing the full length II-III loop are more strongly activated than those lacking R19 (Fig. 6E). Peak inward currents of full-length E-type channels were increased by 100 nM PDBu to 143 ± 4% (n = 12). The simultaneous truncation of both, R19 (Fig. 1) and the homologous segment adjacent to exon 20 (= R20) completely abolishes the phorbol ester mediated stimulation (99 ± 2%, n = 9) [11]. However, the deletion of only insert R19 clearly attenuates the phorbol ester mediated stimulation of peak inward currents but does not reduce it completely (106 ± 2%, n = 13).

The remaining susceptibility for phorbol ester stimulation after deletion of only R19 was investigated more in detail for the splice variant Ca_v2.3e which is

expressed in heart and endocrine tissues. These experiments were performed in equimolar Ca²⁺ instead of Ba²⁺. It is known that the full length Ca_v2.3d splice variant which was stably expressed in HEK293 cells, is maximally stimulated when Ca²⁺ is used as charge carrier [11]. But, the deletion of R19 attenuates both, the kinetics for inactivation [9] and the recovery from inactivation (Fig. 7A) leading to the hypothesis that the deletion of the first arginine-rich segment (R19) might severely reduce the affinity for PKC. To test this hypothesis, exogenous PKC α was coexpressed with Ca_v2.3e in HEK-293 cells using the pPKC α -EGFP vector. The overexpression of PKC α leads to a faster recovery from inactivation in the absence (Fig. 7B) and the presence of phorbol ester (Fig. 7C), in the Ca_v2.3e splice variant lacking R19. This finding supports our assumption that the apparent affinity of the Ca_v2.3e for a PKC interaction differs between those variants lacking or containing R19.

In conclusion, these data lead to the suggestion that

Fig. 5. DSG abolishes the Ni^{2+} -induced increase of the b-wave amplitude recorded from the isolated bovine retina. The isolated bovine retina was equilibrated with nutrient solution to establish a stable response. Subsequently, NiCl_2 was either applied directly (panel A and B) or lactose or DSG (20 μM) was perfused for 40 min, and finally 15 μM NiCl_2 was applied for 50 - 60 min (panel C-F). A.) NiCl_2 mediated (15 μM) stimulation of b-wave amplitude from bovine retina. B.) Original traces for the ERG in panel A as indicated by roman numbers. C.) The b-wave amplitude was increased by 15 μM NiCl_2 under control conditions, and the Ni^{2+} -mediated b-wave response was fully reversible. D.) No significant increase by 15 μM NiCl_2 was observed in the presence of 20 μM DSG. E.) Ratio of basic b-wave amplitude after lactose (left) or DSG application (right). F.) Ratio of b-wave amplitude under 15 μM NiCl_2 related to the subsequent washout by nutrient solution.

both native occurring splice variants are modulated by phorbol esters, but to a different extent.

Autophosphorylation of PKC α is stimulated by the II-III loops of Ca $_v$ 2.3.

According to the literature, Ca $_v$ 2.2 (N-type) α 1 subunits are regulated by PKC dependent phosphorylation of the cytosolic linker connecting domain I and II [24]. Similarly, Ca $_v$ 2.3 currents are potentiated by PKC-dependent phosphorylation, which is stimulated by muscarinic receptor activation. Several potential PKC phosphorylation sites are common between the Ca $_v$ 2.1 and Ca $_v$ 2.2 channels, but some are unique to Ca $_v$ 2.3

leading to the suggestion that Ca $_v$ 2.3 is in addition activated through phosphorylation sites in the carboxy terminal domain of the channel protein [25]. When analysing the phorbol ester mediated stimulation of Ca $_v$ 2.3, we and others [25] found that the II-III - loop is an important determinant for phorbol ester-mediated activation of Ca $_v$ 2.3 recorded as increase in peak inward current (Fig. 6). As phorbol esters activate PKC, we assumed initially a direct phosphorylation of the II-III - loop. However, none of the different constructs of the II-III - loop was phosphorylated by exogenously added PKC α which should have led to phosphoproteins of 62 to 66 kDa (Fig. 8). The only phosphorylated target is a protein of 75 kDa,

Fig. 6. Increase in current amplitude after application of 100 nM PDBu. A – D. Superimposed current traces of Ba²⁺ inward currents before and after stimulation by PDBu for the wt Ca_v2.3 (A.), the mutant lacking both R19 and R20 called Ca_v2.3ΔR19ΔR20 (B.), the mutant lacking only R20 Ca_v2.3ΔR20 (C.), and the endocrine splice variant of Ca_v2.3 which is lacking exon 19 encoded R19 (D.). E. The ratio of peak inward current 2 min after bath application of 100 nM PDBu to that one without phorbol ester is plotted for the neuronal variant, for two different mutants and for the endocrine splice variant Ca_v2.3e lacking exon 19 encoded R19. The full length II-III loop of neuronal Ca_v2.3d is mutated by deletion to Ca_v2.3ΔR19ΔR20 lacking 38 amino acids. The second mutant Ca_v2.3R20 is lacking R20, while the native splice variant Ca_v2.3e is lacking insert R19.

the added PKCα itself, which was detected as a phosphoprotein with an identical molecular size when the eluate from mock transfected cells was added to the phosphorylation assay or when no protein homogenate from HEK-293 cells was introduced to the assay system (Fig. 8B, lane 1 and 11). If any of the four different II-III - loop constructs was used for transfection and the immunopurified protein was added to the phosphorylation assay, the autophosphorylation of the 75 kDa protein, supposed to be PKCα, was dramatically increased. Even more, if no exogenous PKC was added, a polypeptide of

Fig. 7. Application of phorbol ester and over-expression of PKCα influences I_{Ca} carried by Ca_v2.3e. A.) Mean fractional recovery from short term inactivation, observed in 15 mM Ca²⁺ under control conditions (■) and in the presence of 100 nM PDBu (●). B.) Mean fractional recovery from short term inactivation, observed in 15 mM Ca²⁺ under control conditions (■) and after co-transfection of PKCα (Δ). C.) Mean fractional recovery from short term inactivation, observed in 15 mM Ca²⁺ under control conditions (■) and in the presence of 100 nM PDBu after co-transfection of PKCα (○). The number of cells used in panel A to C was 3 to 9.

the same electrophoretic mobility was weakly phosphorylated (Fig. 8, lane 4, 6, 8, and 10), indicating that endogenous PKC co-immunoprecipitates at low amounts with over-expressed II-III - loop segments in HEK-293 cells.

A polypeptide with the same electrophoretic mobility as hsp70 was not detected in our co-immunoprecipitation assays with truncated II-III loops (Fig. 3, lane 4) assuming that the affinity of the tested loops differs dramatically. The data suggest that autophosphorylation of PKCα is highly stimulated by the presence of any of the II-III loop

Fig. 8. The immunopurified FLAG-tagged II-III – loops enhance the autophosphorylation of exogenously added protein kinase C. HEK-293 cells were transfected with four different vectors encoding either the full length II-III – loop of $\text{Ca}_v2.3\text{d}$ (lane 3 and 4), or with mutated loops either lacking exon 19, ΔR19 (lane 5 and 6), or lacking the adjacent arginine-rich segment in exon 20, ΔR20 (lane 7 and 8) or lacking both arginine-rich segments, ΔREL (lane 9 and 10). In parallel, a mock vector was transfected (lane 1, 2), and autophosphorylation was additionally controlled by omitting HEK-293 derived proteins during phosphorylation (lane 11). The incorporation of P-32 radioactivity was measured as an autoradiogram which was either exposed for 6 (panel A) or for 24 hours (panel B). Note, autophosphorylation of exogenously added protein kinase C α in the absence of any II-III – loop constructs is only seen after the longer exposition in panel B (lane 11). – In both panels A and B, exogenous PKC α was only added in lanes 1, 3, 5, 7, 9, 11. Endogenous PKC must be responsible for the phosphoprotein in lanes 2 (see long time exposure), 4, 6, 8, and 10 with the same electrophoretic mobility as exogenous PKC α . This figure shows the results from one out of four experiments.

of $\text{Ca}_v2.3$ in the phosphorylation assay, assuming that stimulation of PKC also occurs *in vivo* by a similar interaction.

In conclusion, hsp70 interacts with the II-III loop of $\text{Ca}_v2.3$, at the biochemical and functional level. Further, PKC α does not directly bind to $\text{Ca}_v2.3$ but it may mediate its stimulatory effect through hsp70, a known PKC-interacting protein [26, 27].

Discussion

In HEK-293 cells, the *in vivo* charge carrier Ca^{2+} modulates at physiological concentrations the kinetics of $\text{Ca}_v2.3$ induced inward currents by Ca^{2+} accumulation inside the cells [10]. This process is different from the Ca^{2+} dependent inactivation, which is also known for $\text{Ca}_v2.3$ [28], and which occurs at more elevated intracellular Ca^{2+} levels. Initially, when Ca^{2+} has entered the cells through $\text{Ca}_v2.3$, it slows down inactivation and fastens recovery from inactivation of the recorded Ba^{2+} inward currents [9, 10]. *In vivo*, the Ca^{2+} dependent modulation of E-type Ca^{2+} channels may lead to a faster recruitment of neuronal $\text{Ca}_v2.3\text{c}$ or $\text{Ca}_v2.3\text{d}$ Ca^{2+} -channels during long term potentiation in the CA3 region of hippocampus [29].

Based on the comparison of *in vivo* occurring splice variants, we found that the novel Ca^{2+} -dependent activation of neuronal $\text{Ca}_v2.3\text{c}$ and $\text{Ca}_v2.3\text{d}$ variants is dependent on structural determinants in the II-III loop, the exon 19 encoded arginine-rich segment (R19) [9]. The Ca^{2+} -sensitivity of $\text{Ca}_v2.3\text{d}$ which leads to the stimulation of I_{Ba} and to the reported changes in kinetics, is strongly attenuated, but not completely abolished, when R19 was removed, as it is accomplished in the so called “endocrine splice variant” $\text{Ca}_v2.3\text{e}$ [15].

The Ca^{2+} -mediated effects on the activity of $\text{Ca}_v2.3$ /E-type channels resemble the effects on $\text{Ca}_v2.3$ mediated by phorbol esters [11]. As observed with increasing cytosolic $[\text{Ca}^{2+}]$, phorbol ester stimulation is also dependent on the exon 19. Therefore we assume, that PKC interacts directly or indirectly with the II-III loop of $\text{Ca}_v2.3$ as it was proposed by Kamatchi and coworkers [25]. Based on the results of the present study, hsp70 binds directly to the neuronal and endocrine II-III loops of $\text{Ca}_v2.3$, and is functionally interacting with retinal neurotransmission. Further, PKC α as a classical Ca^{2+} activated protein kinase does not directly bind to the II-III loop of $\text{Ca}_v2.3$, and one may speculate, that hsp70 mediates the binding of PKC to the II-III loop to support phosphorylation of the channel protein.

The interaction of hsp70 with Ca^{2+} -dependent PKC

Fig. 9. Proposed interaction of hsp 70, protein kinase C, and different variants of the II-III – loop. The model of interaction has to account for at least four different facts: (i) The II-III – loops of both splice variants co-immunoprecipitate with substantial amounts of the hsp70 protein (note, hsp70 is bound to the channel in all four panels). (ii) Only the $\text{Ca}_v2.3$ channel with the full length loop is highly Ca^{2+} -sensitive which is caused by its higher sensitivity towards phorbol esters, and causes its slowing of inactivation and acceleration of recovery from inactivation (situation in panel B). (iii) The chaperone hsp70 is well known to interact with cysteine string proteins as syntaxin, synaptotagmin and SNAP-25 [37]. Synaptotagmin functionally interacts with $\text{Ca}_v2.3\text{d}$ containing E-/R-type voltage-gated Ca^{2+} channels recruiting the protein complex to the plasma membrane of *Xenopus* oocytes which only would be possible according to panel B [34, 35]. Micromolar $[\text{Ca}^{2+}]_i$ inside the cells influences the channel kinetics more potently when $\text{Ca}_v2.3$ subunits containing a full length II-III – loop are expressed in HEK-293 cells [10]. Only when hsp70 interacts with the full length loop, it can keep PKC active for a longer time. The deletion of exon 19 encoded R19 in the endocrine splice variant causes nearly a complete loss of Ca^{2+} sensitivity (panel C and D) which can be overcome by over-expression of $\text{PKC}\alpha$ (not shown).

has been summarized recently [27]. When PKC is activated, it is highly sensitive towards dephosphorylation. Hsp70 is able to bind to the dephosphorylated turn motif of PKC and stabilizes it. PKC becomes rephosphorylated and is able to re-enter the pool of signalling-competent PKC. If hsp70 is missing, dephosphorylated PKC accumulates in a detergent-insoluble cell fraction and is degraded [26, 27].

As hsp70 was shown to co-immunoprecipitate with the II-III - loop of $\text{Ca}_v2.3$ after over-expression in HEK-293 cells, such kind of a PKC stimulation may occur during the phorbol ester and Ca^{2+} mediated modulation of transfected $\text{Ca}_v2.3$ [11] and activated PKC could interact indirectly with the II-III loop of $\text{Ca}_v2.3$ by using the hsp70 as an adaptor protein. The differences of the electrophysiological results observed with the different II-III loop constructs [9-11] could be caused by a different affinity of the two II-III loops to hsp70, which then may indirectly influence the phosphorylation rate of PKC. This assumption is supported by the observation that a 79 kDa protein is mainly found after transfection of the full length but not with shorter variants of II-III loops (see Fig. 2). Also the observation that over-expression of $\text{PKC}\alpha$ changes the kinetics of $\text{Ca}_v2.3\text{e}$ to that of $\text{Ca}_v2.3\text{d}$ (see Fig. 7) points to differences in affinities for interaction

partners of the various II-III loops which can be compensated by a higher expression level of $\text{PKC}\alpha$ in HEK-2C6 cells. Alternate factors may also be considered as for example the competitive interaction with auxiliary subunits [21], although we have shown that the co-transfection of the β -subunit is not necessary to demonstrate the phorbol ester effects on $\text{Ca}_v2.3$ [11].

A direct phosphorylation of the cytosolic II-III loop by PKC probably can be excluded because after *in vitro* phosphorylation of immunoaffinity purified II-III - loop no other major phosphoprotein was detected except the autophosphorylated exogenous $\text{PKC}\alpha$ itself, leading to the suggestion that the hsp70-bound II-III loop increases the activity of PKC by its association. When we compare the extent of the PKC autophosphorylation by the different II-III loops *in vitro*, the truncated II-III - loops stimulate autophosphorylation to the same extent as the full length II-III - loop, suggesting that a selective stimulation of autophosphorylation is not only defined by the II-III - loop structure or depend on the presence or absence of the arginine-rich segment, but also dependent on additional factors. Further, it is known from the literature that the PKC activity is modulated by arginine-rich peptides [30]. The results with hsp70 showing functional interaction, and the effect of purified hsp70 - II-III loop on

autophosphorylation support the idea that additional unknown factors may cause the more selective stimulation of inward currents observed by Ca^{2+} and phorbol esters for the full length neuronal II-III loop of $\text{Ca}_v2.3$ in HEK-293 cells.

Based on the present results and on the reported interactions of $\text{Ca}_v2.3$ with synaptic vesicle proteins, it is presumed that for the full length II-III loop a trimeric complex may associate to activate PKC maximally including an unknown interaction partner X which provides the selectivity of the Ca^{2+} - and PKC-dependent activation of the neuronal II-III loop (Fig. 9A, B). The most favourite candidate as an additional interaction partner is synaptotagmin for three major reasons: (i) it is one of those cysteine string proteins which acts as a known co-chaperone for hsp70 [31], (ii) it is a substrate for PKC [32] and most importantly, (iii) synaptotagmin is that synaptic vesicle protein, which interacts with E-/R-type Ca^{2+} channels in a Ca^{2+} -selective manner [33-35]. The other possible candidate, syntaxin-1A, does not functionally interact with full length $\text{Ca}_v2.3$ in HEK-293 cells [9]. In conclusion, insert R19 may confer an increased Ca^{2+} -sensitivity to $\text{Ca}_v2.3$ through its interaction with synaptotagmin or another partner X (Fig. 9A, B).

The detailed interaction of $\text{Ca}_v2.3$ and hsp70 is by far not yet completely understood and rather speculative for the membrane-bound full-length channel. So far, we can demonstrate, that the II-III loop is an important determinant for PKC signalling, thus phosphorylating other

regions of the E-type Ca^{2+} channel protein for an increased activation, and that hsp70 is an important docking partner involved in this process. As hsp70 is also involved in the modulation of apoptosis, ischemic heart disease, diabetes and neurodegeneration [36], the understanding of this mechanism for E-type Ca^{2+} channel regulation will be important to unravel the recently discovered role of $\text{Ca}_v2.3$ in apoptosis and juvenile myoclonic epilepsy [6, 38].

Abbreviations

$\text{Ca}_v2.3$ (ion conducting $\alpha 1$ subunit of E-type voltage-gated Ca^{2+} channels); DSG (15-deoxyspergualin); ERG (electroretinogram); hsp70 (heat shock 70 kDa protein 1A); PKC (protein kinase C); PDBu (phorbol-12,13-dibutyrate); FLAG (a fusion tag consisting of eight amino acids (DYKDDDDK) including an enterokinase-cleavage site); (c)-myc (a fusion tag consisting of 11 amino acids (EQKLISEEDL)).

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