IV RECI Meeting
New Horizons in Ion Channel Research

February 12-13, 2013
Hotel NH, Cuenca
www.reci-ionchannel.org
Tuesday, 12 February 2013

9:00-9:30 Welcome by the Organizing Committee and local Authorities

Session 1. Dynamic regulation of ion channel assembly and trafficking

Chairman: Antonio Ferrer Montiel (UMH, Elche, Alicante)

9:30 Juan Lerma (Instituto de Neurociencias, CSIC, Alicante)
“The role of NeCaB1 in GluK5 containing kainate receptors trafficking”.

9:50 Francisco Ciruela (Universidad de Barcelona)
“Small-conductance Ca2+ activated K+ channel type 2 and metabotropic glutamate receptor type 5 assemble into functional interacting complexes”.

10:10 Isabel Pérez-Otaño (CIMA, Universidad de Navarra, Pamplona)
“Reactivation of juvenile NMDA receptors triggers synapse loss and motor dysfunction in Huntington’s disease”.

10:30 Antonio Felipe (Universidad de Barcelona)
“Molecular determinants in the Kv1.3 C-terminus are responsible for channel anterograde transport and surface expression”.

10:50 Anna García Elias (Universidad Pompeu Fabra, Barcelona)
“PIP2-dependent rearrangement of TRPV4 cytosolic tails enables channel activation by physiological stimuli”.

11:15 Poster Session 1: Coffee break and posters presentation

Session 2. Ion channels in excitable and non-excitable cells

Chairman: Francisco Ciruela (UB, Barcelona)

12:00 Alfonso Araque (Instituto Cajal, CSIC, Madrid)
“Frequency-dependent transformation of GABAergic inhibition into glutamatergic potentiation in astrocyte-neuron networks”.

12:20 Pedro Grandes (Universidad del País Vasco, Bilbao)
“Subcellular distribution of the transient receptor potential vanilloid type 1 (TRPV1) in the mouse dentate gyrus”.

12:40 José Manuel Juiz (IDINE, Universidad de Castilla-La Mancha, Albacete)
“KCNQ5/Kv7.5 in the auditory pathway: Distinct subcellular localization and regulation by auditory activity”.

13:00 Sara Pagans (Universidad de Girona-IDIBGI, Girona)
“Regulation of the cardiac sodium channel expression and function”.

13:20 Carmina Verdiá Báguena (Universitat Jaume I, Castellón)
“A novel finding for CoV E protein: Coronavirus envelope protein forms ion channels with membrane lipids functionally and structurally involved”.

14:00 Lunch
Session 3. Structure and function of ion channels

Chairman: Oscar Millet (CICbioGUNE, Bilbao)

15:30 Carmen Delgado (Facultad de Medicina, Universidad Complutense, Madrid)
“Leptin up-regulates the transient outward potassium current (I_{to}) through modulation of AKT-signalling in adult rat ventricularmyocytes”.

15:50 José M. Fernández-Fernández (Universidad Pompeu Fabra, Barcelona)
“New molecular determinants for the functional interaction among Ca_V channels and SNAREs”.

16:10 Paula Pluta (CICbioGUNE, Bilbao)
“Hemichannel interactions in connexins: from the computer to the experiment”.

16:30 Marcel Aguilella-Arzo (Universitat Jaume I, Castellón)
“Ionic partition, Ionic transport and selectivity of a protein channel from non-equilibrium MD simulations”.

16:50 Ana Isabel Fernández Mariño (Universidad Pompeu Fabra, Barcelona)
“Molecular characterization of BK channel modulation by tungstate”

17:15 Keynote Lecture

Paul Slesinger (Dept. of Neuroscience, Friedman Brain Institute, Mount Sinai School of Medicine, New York, New York, USA.). Title: Drug-dependent changes in GIRK/Kir3 channel signalling: A new therapeutic target?

18:30 Guided visit to the city of Cuenca.

21:00 Gala Dinner – Restaurante El Parador de Cuenca.

Wednesday, 13 February 2013

Session 4. Pharmacology of ion channels

Chairman: Enrique Pérez Payá (CIPF, Valencia)

9:00 Rosario González Muñiz (IQM-CSIC, Madrid)
Generation of molecular diversity from amino acids. A source for the discovery of new TRP channel modulators.

9:20 Asía Fernández-Carvajal (Universidad Miguel Hernández, Elche)
“High-throughput screening assays for the identification of compounds targeting ion channels”.

9:40 María Queralt-Martín (Universitat Jaume I, Castellón)
“La^{3+}-induced asymmetric current inhibition in OmpF channel”.

10:00 Eva Delpón (Universidad Complutense, Madrid)
“Pharmacological modulation of cardiac inward rectifier channels. A new challenge”.

10:20 Rosa Planells (Max-DMM, Berlin, Germany)
“Potentiation of the Transient Receptor Potential Vanilloid 1 (TRPV1) channel contributes to pruritogenesis in a rat model of liver disease”.

12-13 February 2013
10:45  **Poster Session 2**: Coffee break and posters presentation

**Session 5. Ion channels in health and disease**

Chairman: **Isabel Pérez-Otaño** (CIMA, Pamplona)

11:30  **Felix Viana** (Instituto de Neurociencias, CSIC-UMH, Elche)  
“Targeting TRP channels for the relief of cold discomfort and cold pain”.

11:50  **Teresa Giráldez** (Universidad de la Laguna, Tenerife)  
“The neuronal serum- and glucocorticoid-regulated kinase 1.1 reduces neuronal excitability and protects against seizures through upregulation of the M-current”.

12:10  **Raúl Estévez** (Universidad de Barcelona-CIBERER, Barcelona)  
“Analysis of a MLC1 KO mouse models provide new insights in the pathophysiology of the leukodystrophy MLC: Chloride channels are not working properly”.

12:30  **Antonio Alcaraz** (Facultad de Biología, Universidad Complutense, Madrid)  
“Hydrophobic pulmonary surfactant proteins SP-B and SP-C induce pore formation in planar lipid membranes: evidence for proteolipid pores”.

13:00  **Keynote Lecture**

**Jean-Marc Fritschy** (Director of the Neuroscience Center Zurich, Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland).  
**Title**: “Role of GABA_4 receptors in regulating adult neurogenesis”.

14:00  **Lunch, concluding remarks and farewell.**
ORGANIZATION

Scientific Committee
Ricardo Borges (ULL, Tenerife)
Francisco Círuela (UB, Barcelona)
Antonio Ferrer Montiel (UMH, Elche, Alicante)
Rosario González Muñiz (Instituto de Química Médica-CSIC, Madrid)
José M. Juiz Gómez (IDINE-UCLM, Albacete)
Juan Lerma (Instituto de Neurociencias, Alicante)
Rafael Luján (IDINE-UCLM, Albacete)
Isabel Pérez Otaño (CIMA, Pamplona)

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KEYNOTE LECTURES
Paul Slesinger

Dept. of Neuroscience, Friedman Brain Institute, Mount Sinai School of Medicine, New York, New York, USA

Natural reward and addictive drugs activate dopamine (DA) neurons in the reward pathway that originate in the ventral tegmental area (VTA). In general, addictive drugs co-opt the brain’s intrinsic reward system, and there is considerable interest in understanding the long-term changes in the neural circuitry that occur with addictive drugs. In the DA and GABA neurons of VTA, GABA_B receptors coupled to G protein-gated inwardly rectifying potassium (GIRK) channels comprise a prominent inhibitory pathway. Our laboratory has been investigating mechanisms of plasticity in GABA_B-GIRK signaling in the VTA that occur with addictive drugs. Recently, we discovered that an acute injection of psychostimulant in mice produces a profound depression of GABA_B-GIRK signaling that is evident 24h after the injection, well after the drug has cleared the body, and remains depressed for up to 7 days later. This depression is observed in VTA GABA neurons and not in VTA DA neurons or GABA neurons of the hippocampus and prelimbic cortex. We have investigated the biochemical pathways involved in mediating the effect of psychostimulants in GABA neurons, and discovered a key role for a protein phosphatase. The persistent reduction in GABA_B-GIRK signaling in the VTA suggests there is a cellular memory trace that is encoded in the phosphorylation status of the GABA_B receptor. We also have new data revealing the role of a novel cytoplasmic regulator of GIRK channels, sorting nexin 27, in controlling GABA_B-GIRK currents in the VTA. Together, these findings illustrate new ways to modify activity of neurons in the reward pathway, pointing toward new types of treatments for addiction.
GABA$_A$ receptors, which mediate most fast synaptic inhibition in the CNS, form pentameric ligand-gated ion channels permeable for Cl$^-$ and HCO$_3^-$ ions. They are encoded by a large family of subunit genes, which are differentially expressed and assembled in multiple GABA$_A$ receptor subtypes. In particular, the six alpha subunit variants (alpha1-alpha6) define receptors with distinct functional and pharmacological properties. Thus, the alpha4 and alpha6 subunits form receptors insensitive to classical benzodiazepines, such as diazepam, and located predominantly at extrasynaptic sites. The alpha5 subunit also corresponds mainly to extrasynaptic receptors and is highly abundant in the hippocampal formation.

Multiple lines of evidence indicate that GABAergic transmission regulates multiple steps of neurogenesis in the developing and adult brain, ranging from proliferation of neural progenitor cells (NPC) to migration, dendritic differentiation and synaptic integration of newborn neurons, raising the possibility that these various effects are mediated by distinct GABA$_A$ receptor subtypes, in particular those mediating tonic inhibition at extrasynaptic sites. Using targeted gene deletion approaches, we have shown that alpha4-GABA$_A$ receptors negatively regulate NPC proliferation in adult brain and control migration and early dendritic differentiation of dentate gyrus granule cells. In contrast, alpha2-GABA$_A$ receptors, which are located selectively at postsynaptic sites, are important for late dendritic maturation and synapse formation. Recent evidence points to a role for alpha5-GABA$_A$ receptors in controlling division and self-renewal mode of slowly proliferating nestin-positive stem cells, thereby regulating the size of the NPC pool (Song et al, 2012, Nature 489, 115). These receptors also determine cell fate decision, favoring neuronal over astrocytic differentiation.

Taken together, these results indicate that the functional specialization of GABA$_A$ receptors by means of multiple subunit genes is of major significance for the regulation of adult neurogenesis in the CNS.
ORAL PRESENTATIONS
The Role of NeCaB1 in GluK5 Containing Kainate Receptors Trafficking

J. Palacios-Filardo, Rocio Rivera and J. Lerma.

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Fast excitatory synaptic transmission is mainly mediated by glutamate receptors in the Central Nervous System (CNS). This family of receptors comprises three different subfamily named after ligand preference: a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainate. Among these, kainate receptors (KARs) are the less understood from a physiological point of view. An attempt to unveil important aspects of KARs physiology is to elucidate the protein interactome around these receptors. To reach this goal, our lab used a yeast two-hybrid screening to identify possible partners of GluK5 subunits by using its C-terminal domain (CTD) as a bait. During this screening, we identified Neuronal Calcium Binding Protein 1 (NeCaB1) as an interactor of GluK5 CTD. We further verified the interaction between NeCaB1 and GluK5 by co-immunoprecipitation in HEK cells expressing both proteins and in by pull-down assay from native tissue. In addition, we found that binding of NeCaB1 to GluK5 CTD is calcium dependent in that interaction is disfavored in the presence of calcium. Bimolecular fluorescence complementation (BiFC) further demonstrated interaction between these two proteins in vivo. This interaction occurs in specific CTD regions that contain endoplasmatic retention signals, likely indicating a role in receptor trafficking.

The increased affinity for glutamate of GluK1/GluK5 heteromeric KARs as compared to homomeric GluK1 receptors served as a readout for detecting GluK1/5 heteromeric receptors at the plasma membrane. We found that NeCaB1 promotes the presence of GluK5 containing KARs in the cell surface when internal calcium was reduced to a minimum. Furthermore, these trafficked receptors present a higher affinity under conditions promoting interaction with NeCaB1 (e.g. low Ca$^{2+}$). Altogether, these data indicate that NeCaB1 binds to CTD of GluK5 subunit containing KARs promoting its trafficking to the cell surface and increasing their sensitivity to the agonist in a low calcium environment. This dual mechanism may represent a sort of homeostatic plasticity in synapses where KARs are present.

Supported by the Spanish MICINN (BFU2006-07138 and BFU2011-24084), CONSOLIDER (CSD2007-00023) and Prometeo/2011/086. JP-F was supported by a Ph.D. fellowship from the Basque Government.
The small-conductance Ca\(^{2+}\) activated K\(^{+}\) channel type 2 (SK\(_{2}\)) are exclusively activated by intracellular Ca\(^{2+}\) ions. Its activation controls the somatic excitability by contributing to the after-hyperpolarization phenomenon, modulates synaptic plasticity by coupling to NMDA receptors and influences dendritic Ca\(^{2+}\) levels. Recently, it has been demonstrated that group I metabotropic glutamate receptors (i.e., mGlu5 receptors) regulate hippocampal CA1 pyramidal neuron excitability via Ca\(^{2+}\) wave-dependent activation of SK\(_{2}\) channels. However, the precise functional and molecular receptor-channel interaction still unsolved. Here we demonstrated that SK\(_{2}\) channels and mGlu5 receptors interact both in heterologous expression systems and in native tissue (i.e., rat hippocampus). Accordingly, both proteins co-distributed at the subcellular level in rat hippocampus and also co-immunoprecipitated from rat hippocampal membranes. In addition, we confirmed this interaction in transiently transfected HEK293 cells by means of co-immunoprecipitation, BRET and FRET experiments. Interestingly, co-transfection of SK\(_{2}\) channels with mGlu5 receptors promoted cell surface expression of both proteins. This increment in mGlu5 receptor plasma membrane expression correlated well with an increased receptor function, an effect precluded by incubation with the SK2 channel blocker apamin (300 nM). On the other hand, in HEK293 cells expressing both proteins the incubation with L-glutamate (1 mM) enhanced K\(^{+}\) inward current, and effect that was blocked with apamin. Interestingly, HEK293 cells expressing only SK2 channels didn’t show this L-glutamate sensitivity. Overall, these data demonstrated that a functional and molecular interaction occurs between SK2 channels and mGlu5 receptors at the plasma membrane level and suggested that the mGlu5 receptor might control the SK2 channel gating under certain physiological circumstances (i.e. sustained glutamate release conditions).

**Small-conductance Ca\(^{2+}\) activated K\(^{+}\) channel type 2 and metabotropic glutamate receptor type 5 assemble into functional interacting complexes**

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Supported by grants SAF2011-24779 and Consolider-Ingenio CSD2008-00005 from Ministerio de Ciencia e Innovación and ICREA Academia-2010 from the Catalan Institution for Research and Advanced Studies to FC.
Rescue of NMDAR dysfunction, spine loss and motor symptoms in HD models by suppressing aberrant GluN3A expression


GluN3A and GluN3B are non-conventional members of the NMDAR subunit family that modulate NMDAR-dependent synaptic plasticity by altering the calcium permeability and magnesium sensitivity of the receptor complexes. GluN3A subunits are highly expressed in the brain during critical periods of postnatal development but down-regulate sharply afterwards. Here we will present recent findings showing that adult reactivation of non-conventional GluN3A expression in Huntington disease (HD) causes synapse dysfunction and loss. Specifically, we found that sequestration of the GluN3A-selective endocytic adaptor PACSIN/syndapin 1 by mutant huntingtin causes a pathological accumulation of juvenile NMDARs at the surface of striatal neurons, both in cellular and mouse models and in human HD striatum. GluN3A subunits play critical roles in preventing premature synapse stabilization and maturation in developing brains, but switching back their expression in the adult is deleterious and triggers synapse loss. Remarkably, reversing aberrant GluN3A expression prevented the earliest signs of synapse degeneration, ameliorated motor and cognitive impairment, and reversed abnormally increased NMDAR currents in the YAC128 model of HD. Our work identifies a novel disease mechanism that underlies the pathology of HD, and identifies the huntingtin interactor PACSIN1 as the missing link between mutant huntingtin and dysregulation of NMDARs.
Molecular Determinants in the Kv1.3 C-Terminus Are Responsible for Channel Anterograde Transport and Surface Expression


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The Kv1.3 channel is crucial in sensory neurons and leukocytes. The anterograde transport and membrane expression of Kv1.3 is altered in autoimmune diseases and deficient axonal targeting. Unexpectedly, molecular mechanisms underlying Kv1.3 channel trafficking remain elusive. We identified a noncanonical Y479MVIEE484 motif at the Kv1.3 C-terminus, notably conserved in neuronal Kv1 channels, which triggers the anterograde transport and surface expression. Within this cluster, a specific di-acidic (E483-E484) motif was essential for interactions with the Sec24 subunit of the coat protein complex II (COPII). A molecular model of the Kv1.3-Sec24a complex indicated that Y479MVIEE484 was a water-exposed loop. Further, this E483/484 motif strongly interacted with R750/752 in Sec24 through salt bridges. These trafficking motifs contribute to our understanding of the mechanisms underlying the targeting of channels to the cell membrane and can aid to interpret channel function under physiological and pathological conditions.

Supported by BFU2011-23268 to AF, BFU2009-08346 to AFM, PROMETEO2010/046 to AFM and CSD2008-00005 to AF and AFM
**Objectives**: Most TRP channels are regulated by PIP2, although the structural rearrangements occurring upon binding of this lipid are currently far from being understood. In the present study we report that TRPV4 activation by hypotonic and heat stimuli requires PIP2 binding to and rearranging of the cytosolic tails.

**Results**: Neutralization of the positive charges or replacing the aromatic residue within the TRPV4 N-tail sequence 121KRWRK125, which resembles a phosphoinositide binding site (PI-site), rendered the channel unresponsive to hypotonicity and heat but did not affect its response to 4α-PDD, a synthetic agonist that binds directly to transmembrane domains.

Similar channel response was obtained by depleting PIP2 from the plasma membrane with translocatable phosphatases in heterologous expression systems or by activation of phospholipase C in native ciliated epithelial cells.

In inside-out patches, PIP2 facilitated TRPV4 activation by the osmotransducing cytosolic messenger 5'-6'-EET and allowed channel activation by heat.

Protease protection assays demonstrated a PIP2 binding site within the N-tail, further supporting the role of PIP2 in TRPV4 regulation.

The proximity of TRPV4 tails, analysed by FRET, increased by depleting PIP2, by mutations in the PI-site or by co-expression with PACSIN3, a regulatory molecule that binds TRPV4 N-tails and abrogates activation by cell swelling and heat. PACSIN3 lacking the membrane-anchoring F-BAR domain interacted with TRPV4 without affecting channel activation or tail rearrangement.

**Conclusions**: Therefore, mutations weakening the TRPV4-PIP2 interacting site and conditions that deplete PIP2 or difficult TRPV4 access to PIP2 –“a foot in the door” scenario in the case of PACSIN3- change tail conformation and negatively affect channel activation by physiological stimuli hypotonicity and heat.
Frequency-Dependent Transformation of Gabaergic Inhibition into Glutamatergic Potentiation in Astrocyte-Neuron Networks

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Objectives: Accumulating evidence indicate the existence of bidirectional communication between astrocytes and neurons. We have investigated the response of astrocytes to activity of GABAergic interneurons and the consequent effects on CA3-CA1 glutamatergic synaptic transmission.

Material and Methods: Using electrophysiological and Ca^{2+} imaging techniques in murine hippocampal slices (P12-P18), we performed paired recordings from GABAergic interneurons and pyramidal neurons in the CA1 area, while simultaneously monitoring intracellular astrocyte Ca^{2+} levels. We stimulated Schaffer collateral single synapses using the minimal stimulation method to quantify the synaptic transmission properties.

Results: Depolarization-evoked single action potentials in interneurons induced a transient inhibition of synaptic efficacy and probability of release at single CA3-CA1 synapses. This transient inhibition was abolished by picrotoxin, indicating that it is mediated by activation of presynaptic GABAa receptors. In contrast, trains of action potentials evoked by long depolarizations (500-700 ms) transiently increased the synaptic efficacy and the probability of release at the same single synapses. This phenomenon was abolished by GABAb and group I mGlu receptor antagonists (CGP-55845 and MPEP+LY-367385, respectively), and was associated with elevations in the intracellular Ca^{2+} levels of the surrounding astrocytes. Both the GABA-induced astrocyte calcium elevations and the synaptic potentiation were absent in the IP_3-R2 knockout mice (in which G-protein-mediated astrocyte Ca^{2+} signal is impaired), indicating that high frequency interneuron activity activates GABAb receptors in astrocytes, elevating their Ca^{2+} levels and stimulating the subsequent release of glutamate, which potentiates synaptic transmission by the activation of presynaptic group I metabotropic glutamate receptors at Schaffer collaterals.

Conclusions: Astrocytes decode interneuron activity to induce frequency-dependent differential modulation of synaptic transmission. As a consequence of this property, astrocytes transform in a frequency-dependent manner GABAergic inhibition into potentiation of glutamatergic excitatory synapses.

Supported by Marie Curie (G.P.) and Juan de la Cierva (R.G.) Programs, and Cajal Blue Brain and MICINN (BFU2010-158432 and CONSOLIDER [CDS2010-00045]) grants (A.A.).
Subcellular distribution of the transient receptor potential vanilloid type 1 (TRPV1) in the mouse dentate gyrus

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The endocannabinoid anandamide, in addition to cannabinoid receptors, also activates the transient receptor potential vanilloid type-1 (TRPV1), a nonselective cation channel. In the hippocampal dentate molecular layer, TRPV1 activation by anandamide triggers a non-cannabinoid dependent long-term depression (LTD) upon medial perforant path stimulation (Chávez et al., 2010). Despite these physiological findings, nothing is known about the subcellular distribution of TRPV1 in the dentate molecular layer.

Objective: To investigate the ultrastructural localization of TRPV1 in the dentate molecular layer.

Material and Methods: Specific TRPV1 antibodies were applied to hippocampal vibrosections obtained from perfusion-fixed brain mice (wild-type and TRPV1 knockout). The antibodies were combined with a very sensitive pre-embedding immunogold method for high resolution electron microscopy.

Results: TRPV1 immunoparticles were mostly localized in postsynaptic compartments, particularly in dentate granule cell dendritic spines (44.83%±7.50%) and shafts (29.02%±4.05%) distributed throughout the outer 2/3 of the molecular layer. In addition, only about 10% of the synaptic terminals were TRPV1-immunolabeled in this target region of the glutamatergic perforant path. TRPV1 in the inner 1/3 of the molecular layer, the main target of the excitatory mossy cell axon terminals, was poorly expressed (positive spines: 12.83%±5.52%; dendrites: 17.27%±3.50%; terminals: 4.33%±1.89%). TRPV1 immunolabeling disappeared in the TRPV1-KO dentate molecular layer, meaning that the antibody used was highly specific. TRPV1-KO mice were confirmed by genotyping techniques.

Conclusion: So far, this is the first evidence showing the precise subcellular distribution of TRPV1 in the dentate molecular layer where TRPV1 plays a role in a long-term form of synaptic plasticity.

REFERENCE


Supported by GIC07/70-IT-432-07, SAF2009-07065 and RETICS RD07/0001/2001 (P. Grandes laboratory); and by PNSD 20091039 and RETICS RD06/0001/0002 (Instituto Carlos III and FEDER Funds) (E. Fernández-Espejo laboratory). The authors thank Eduardo Muñoz (Universidad de Córdoba) for the initial gift of TRPV1-KO mice.
KCNQ5/Kv7.5 in the Auditory Pathway: Distinct Subcellular Localization and Regulation by Auditory Activity

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Using PCR and high resolution immunocytochemical methods, we have found that Kv7.5/KCNQ5, a low-threshold, non-inactivating voltage-gated potassium channel is preferentially targeted to excitatory endings of auditory neurons in the rat brainstem. This distinct localization predicts previously unexpected roles for this potassium channel in the regulation of presynaptic excitability and transmitter release. Such a specific targeting to excitatory endings is attained during postnatal development at the time of hearing onset. This suggests that the subcellular compartmentalization of this channel depends on neuronal activity. Elimination of auditory nerve activity by cochlea removal during development prior to hearing onset leads removal of the channel from synaptic endings originating from neurons deprived of activity, as seen by immunocytochemistry. Elimination of auditory nerve activity by bilateral cochlear ablation or intracochlear injection of tetrodotoxin (TTX) in the adult animal, also interfered with the synaptic localization of KCNQ5 in neurons of the auditory brainstem. Hence, several weeks after cochlea removal, KCNQ5 immunoreactivity was much less intense or undetectable in its normal location in calyces of Held in the medial nucleus of the trapezoid body (MNTB), whereas it seemed to redistribute to cell bodies in the anteroventral cochlear nucleus (AVCN), some of which are the origin of MNTB endings. Results were similar a few hours after intracochlear TTX, with much less KCNQ5 immunostaining in endbulbs of Held in AVCN and calyces of Held in MNTB. No changes in KCNQ5 expression were detected in the cochlear nucleus by Western Blot or quantitative RT-PCR, suggesting that removal of KCNQ5 from calyces of Held was not due to gene or protein synthesis down-regulation at their parent cell bodies. We conclude that neuronal activity from auditory nerve afferents is required to maintain the subcellular distribution of this potassium channel in synaptic endings of the auditory brainstem and that removal of the channel from synaptic endings maybe an adaptive mechanisms to adjust neuronal excitability to altered inputs.

Supported by MICIIN BFU2009-13754-C02-01 and PEII09-0152-6233 (JCCM).
Regulation of the Cardiac Sodium Channel Expression and Function

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The α subunit of the cardiac voltage-gated sodium channel (Na\textsubscript{\textalpha},1.5), encoded by the SCN5A gene, is responsible for the rapid influx of sodium ions that initiate the propagation of the action potential in cardiomyocytes. Genetic alterations in SCN5A are the most common cause of Brugada Syndrome (BrS), a life-threatening arrhythmogenic disease associated with a high risk of Sudden Cardiac Death. We are interested in understanding the molecular mechanisms that regulate SCN5A expression and function both at the transcriptional and posttranslational level, and how alteration of these mechanisms contribute to cardiac arrhythmias.

At the transcriptional level, we are examining the role of transcription factors that regulate SCN5A promoter function. We have performed luciferase reporter experiments in cardiac cell lines and observed that the cardiac transcription factor GATA-4 acts as a specific transcriptional activator of SCN5A promoter. We are also performing a comprehensive analysis of the epigenetic marks present on the SCN5A promoter to analyze their role in the regulation of SCN5A expression.

At the posttranslational level, we are studying the modifications that regulate Na\textsubscript{\textalpha},1.5 function. Using a proteomic approach, we recently identified arginine methylation of residues R513, R526 and R680 as a new post-translational modification of Na\textsubscript{\textalpha},1.5 (Swiss-Prot Q14524) (I). Of note, mutations R526H and R680H have been associated with cardiac arrhythmia syndromes. We have performed FRET analysis between Na\textsubscript{\textalpha},1.5 and arginine methyltransferases (PRMTs) and found that PRMT3 and PRMT5 interact with Na\textsubscript{\textalpha},1.5. PRMT3 and PRMT5 also changed Na\textsubscript{\textalpha},1.5 activity in patch-clamp experiments. Our results suggest that PRMT3 and PRMT5 methylate Na\textsubscript{\textalpha},1.5 \textit{in vivo}, thereby regulating Na\textsubscript{\textalpha},1.5 function. Our findings provide novel insights into novel mechanisms on the cardiac sodium channel regulation, which will ultimately contribute to further understanding of cardiac arrhythmias.

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Supported by:

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A Novel Finding for CoV E Protein: Coronavirus Envelope Protein Forms Ion Channels with Membrane Lipids Functionally and Structurally Involved

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Coronaviruses (CoV) are pathogens that cause common colds, bronchiolitis and acute respiratory distress syndrome. In fact, their relevance increased when the causative agent of the severe acute respiratory syndrome (SARS) was identified as a CoV.

Coronavirus (CoV) envelope (E) protein ion channel activity was determined by electrophysiological measurements in planar lipid bilayers by peptides representing either the transmembrane domain of severe acute respiratory syndrome CoV (SARS-CoV) E protein, or the full-length E protein. We have reported that E protein conductance and ion selectivity were controlled by the lipid composition of the membrane [2].

Here we provide additional evidences of the functional involvement of lipids in the channel structure. The influence of lipid molecules on E protein channel transport properties was investigated focusing on the salt concentration dependence of the E protein conductance and the pH dependence of the channel ion selectivity.

The channel conductance in neutral bilayers increased with the electrolyte concentration whereas in charged bilayers it is approximately proportional to the square root of salt concentration, which reveals an electrostatic contribution from the lipid charge. In regard to pH dependence of ion selectivity, in uncharged bilayers the titration curve shows a single transition that corresponds to E protein residue titration, whereas in charge bilayers a second transition is observed, which presumably corresponds to lipid groups titration.

These results support the previous hypothesis that the lipids are functionally involved in E protein ion channel activity, forming a protein-lipid pore, a novel concept for CoV E protein ion channel entity.

Leptin Up-Regulates the Transient Outward Potassium Current (I_{tof}) Through Modulation of Akt-Signalling in Adult Rat Ventricularmyocytes

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Objectives: The adipokine leptin is increased in obesity. Hyperleptinaemia has been confirmed to be a predictor of acute cardiovascular events. However, some studies have shown that leptin has a cardioprotective effect in leptin-deficient models. Nowadays, the mechanisms involved in the cardiac actions of leptin remain poorly understood.

The fast transient outward potassium current (I_{tof}) has an important contribution to the phase 1 of repolarization and action potential duration (APD) in the heart. I_{tof} down-regulation and APD prolongation has been consistently reported in cardiac disease. No information is currently available analyzing the influence of leptin on cardiac channel remodelling. In the present study, we have hypothesized that leptin, could modulate I_{tof} in adult rat ventricular myocytes.

Methods: Enzymatically isolated adult rat ventricular myocytes were treated with 100 ng/mL leptin or vehicle for 48h. Western-blot techniques were used to analyze the protein expression of the molecular components of I_{tof}: Kv4.2 and Kv4.3. The electrophysiological study of I_{tof} was performed by using the whole-cell patch-clamp technique.

Results: Leptin treatment induced an up-regulation of Kv4.2 and Kv4.3 protein expression in ventricular myocytes. In addition, the electrophysiological study showed that both I_{tof} amplitude and density were enhanced by leptin. Previous studies had reported the participation of PI3K/Akt signalling in the up-regulation of K⁺-channels during physiological hypertrophy. In the present study we have demonstrated that leptin activates Akt pathway in cardiomyocytes and this mechanism is involved in the effect of leptin on I_{tof}.

Conclusion: We have demonstrated that leptin through Akt signalling, increases both the expression and function of Kv4.2 and Kv4.3 subunits in adult ventricular myocytes. Altogether these data suggest that leptin may have a beneficial effect by compensating the reduced K⁺ repolarizing reserve that occurs in obese patients with congestive heart failure or coronary heart disease.

Supported by SAF-2010-16377
New Molecular Determinants for the Functional Interaction among Ca\(_\text{v}\) Channels and SNAREs.

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**Aim:** To optimize the exocytotic release of neurotransmitters, several SNARE proteins specifically interact with Ca\(_\text{v}\)2 channels via a synaptic protein interaction site (synprint) located within the intracellular loop connecting domains II and III (LII-III) of the channels, although the involvement of other molecular domains has been proposed. There are increasing evidences supporting a unique role of Ca\(_\text{v}\)3 channels in fast and low-threshold exocytosis, but the underlying molecular mechanisms have remained unknown. Our aim was to further elucidate the molecular determinants and mechanisms involved in the functional interaction among voltage-gated Ca\(^{2+}\) channels and SNAREs.

**Methods:** We have employed cell culture, molecular biology techniques, heterologous expression of WT and mutant voltage-gated Ca\(^{2+}\) channels, and electrophysiological techniques for the recording of both Ca\(^{2+}\) currents and changes in cell capacitance.

**Results:** The regulation of Ca\(_\text{v}\)2.1 (P/Q-type) channels by syntaxin-1A involves also the first intracellular loop of the pore-forming \(\alpha_{1}\text{A}\) channel subunit and requires the lack of a Ca\(^{2+}\)-dependent component in the channel steady-state inactivation. Syntaxin-1A interacts with the carboxyl terminus domain of Ca\(_\text{v}\)3.2 (T-type) channels and modulates both channel activity and low-threshold exocytosis.

**Conclusions:** P/Q and T voltage-gated Ca\(^{2+}\) channels are functionally regulated by syntaxin-1A in a similar manner. Such modulation requires channel structural determinants beyond the synprint site. Disruption of the interaction among Ca\(_\text{v}\) channels and SNAREs, compromise the coupling efficiency of these channels to exocytosis.

Supported by Spanish Ministry of Science and Innovation, Fondo Europeos de Desarrollo Regional (FEDER) Funds, and Plan E [Grants SAF2009-13182-C03-02, SAF2009-09848], Fondo de Investigación Sanitaria [Redes HERACLES RD06/0009] and Generalitat de Catalunya [Grant 2009SGR1369]. MAV is the recipient of an ICREA Academia Award (Generalitat de Catalunya).
Intercellular communication is most essential for multicellular organisms since ion and small molecule exchange are critical for the maintenance of tissue/organ homeostasis. Gap junctions are highly specialized membrane structures used for intercellular communication. Connexins belong to gap junctions with more than twenty of them identified. From the structural point of view, connexins share structural topology: four transmembrane domains forming the hemichannel pore, two extracellular loops associated to cell–cell recognition and the cytoplasmic moieties (N and C termini plus the cytoplasmic loop) which are believed to have regulatory roles. Mutations in genes carrying connexins cause a variety of genetic disorders, such as skin disorders, hearing loss or epilepsy. However, the structural bases for such pathologies are poorly understood.

Based on the crystallized structure of the connexin 26 (Cx26, 2ZW3, 3.5Å resolution) and using homology modelling (SWISS-MODEL), a set of structural models for connexins has been created: Cx26 & Cx26 N176D, Cx32 & Cx32 N175D, Cx47 & Cx47 Y47H H252D. The protein–protein docking method (ClusPro Server), the knowledge of crucial residues (1) and access to experimental data provided by the group of Luis C. Barrio brought us closer to a better understanding of their structure-function relationships. Such results will be explained in the present communication.

(1) Structure of the connexin 26 gap junction channel at 3.5 Å resolution, Maeda S, Nakagawa S, Suga M, Yamashita E, Oshima A, Fujiyoshi Y, Tsukihara T., Nature, 2009 Apr 2;458(7238):597-602

In this talk, we present extensive molecular dynamics (MD) simulations of a multi-ionic protein channel, the OmpF channel, under an external electric field. OmpF is a mesoscopic size channel used routinely as a model system to study ion permeation. We study the ionic current due to monovalent and divalent electrolyte and the affinity of the channel for the different ions, as measured by the ionic partition.

As a result of these simulations under applied electric field, we observe that the current in the OmpF channel is due to cations in the case of KCl solutions but it is due to anions in the case of MgCl2 solutions[1]. In this sense, the selectivity (as measured by the current transported by the channel) is reversed. However in both cases the negatively charged OmpF channel has strong affinity for cations, as revealed by the ionic partition. Divalent cations strongly bind to specific residues located along the channel, being practically immobilised.

Further simulations considering certain OmpF mutants also emphasize the conceptual difference between the affinity of the channel for ions and transport properties. These mutations are able to exclude cations from the channel constriction (present in the central part of the protein), significantly reducing the cationic current as compared to the wild type OmpF protein, while maintaining the anionic part of the current. However, the partition of ions relates fairly well to the total charge of the protein, which is negative in all cases. Again, channel charge and ionic partition are therefore unable to adequately represent the selective properties of the channel under external fields [2].

Overall, our results emphasize the need for non-equilibrium simulations under a electric field in order to understand the selectivity of ionic channels.

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Molecular Characterization of BK Channel Modulation by Tungstate.

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Aim: Tungstate reduces blood pressure in experimental animal models. Given that the large-conductance voltage- and Ca²⁺-dependent K⁺ (BK) channel is a key element in the control of arterial tone, our aim was to evaluate whether BK channel is modulated by tungstate and to identify the channel molecular determinants contributing to such regulation.

Methods: We employed cell culture, molecular biology techniques, heterologous expression of WT and mutant BK channel subunits, and electrophysiological techniques for the recording of K⁺ currents.

Results: We found that tungstate reduces the voltage for channel half-activation (~20 mV) only for BK channels containing αβ₁ or αβ₄ subunits. The β₁-dependent activation of BK channels by tungstate was enhanced at high cytosolic Ca²⁺ levels of physiological relevance. The analysis of available structural data on tungstate-protein complexes supported the role of divalent cations, such as Mg²⁺, as cofactors for tungstate binding. These studies also allowed us to suggest putative tungstate binding sites at the BKα subunit based on an identified aspartate-lysine/lysine-aspartate motif, including the two regions contributing to the Mg²⁺ binding site. Consistently, the activation of BKαβ₁ channels by tungstate was prevented either by removal of cytosolic Mg²⁺ or by α subunit mutations rendering the channel Mg²⁺-insensitive. We have also discovered that mutation of β₁ loop residues involved in the voltage sensor activation and gating of BK channels, strongly impairs tungstate action on BKαβ₁ channels.

Conclusion: BK channel modulation by tungstate involves residues at both BK α and β₁ subunits of relevance for voltage sensor activation.

Supported by Spanish Ministry of Science and Innovation, Fondos Europeos de Desarrollo Regional (FEDER) Funds, and Plan E [Grants SAF2009-13182-C03-02, SAF2009-09848], Fondo de Investigación Sanitaria [Redes HERACLES RD06/0009] and Generalitat de Catalunya [Grant 2009SGR1369]. MAV is the recipient of an ICREA Academia Award (Generalitat de Catalunya).
Generation of Molecular Diversity from Amino Acids. A Source for the Discovery of New TRP Channel Modulators


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Ion channels are central and challenging targets in medicinal chemistry but, because of the scarce structural knowledge, rational approaches to ion channel modulators are still rare. Moreover, the multimodal activation of some channels, like TRPs, complicates still more the scenario for rational discovery programs. Due to these facts, most strategies directed to identify ion channel modulators rely on the screening of peptide and small-molecule libraries.

In this context, we have been involved in the development of synthetic pathways for the generation of diverse, chiral, highly functionalized linear and heterocyclic scaffolds from amino acids, and in the production of discrete libraries from them. The screening of these libraries on different TRP channels has allowed the discovery of some innovative hits that have progressed to hit-to-lead optimization programs. This communication will deal with the synthesis, structural characterization, and biological evaluation of a collection of \( \beta,\gamma \)-diaminoester derivatives that display significant activity at TRPV1, TRPM8 and TRPA1 channels. Compound RGM04-7, a selective TRPV1 blocker, has been selected for \textit{in vivo} studies in rodents.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{RGM04-7}
\caption{RGM04-7}
\end{figure}

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Regulated and selective transport of ions mediates by ion channels underlying numerous fundamental physiological processes. This goes from electrical signal in the heart and the nervous system to hormone secretion or immune response. The physiological importance of ion channels is underlined by their involvement in a wide range of pathologies spanning all mayor therapeutic areas.

Given their critical biological roles it is not surprise that ion channels are a major class of drug targets. Almost all of the current ion channel drugs were discovered using traditional tissue or animal based pharmacological methods, but these processes take a lot of time. There is a need for development of biological relevant assays with the capacity for randomly screening sizeable compound libraries.

Different screening formats have been development using ion or voltage sensitive fluorescent dyes but they lack the precision, temporal resolution and voltage control required for monitoring channel modulation.

On the other hand traditional electrophysiology is too slow, technically demanding and labor intensive for primary screening. Recently these limitations have been addressed by the development of automatically electrophysiology instruments. While retaining much of the fidelity and precision of electrophysiology these system also address the main disadvantages by using automation to increase throughput and deskilling the process.

Here we present the results obtained using a platform for high-throughput screening that has been implemented in the IBMC in which we have combined several techniques for discover better therapeutic ion channel modulators.

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Supported by MICINN grants, Consolider-Ingenio 2010 (CSD2008-00005), UMHE10-3E-409, 2011-2012 and BFU2012-39092-C02-01.
La$^{3+}$-Induced Asymmetric Current Inhibition in OmpF Channel

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OmpF is a general diffusion porin located at the outer membrane of *Escherichia coli*. Its aqueous pore has an hour-glass shape with a narrow constriction placed at about half of the channel pathway. Two acidic residues in this region face a cluster of three positive arginines, creating a high transverse electric field. This separates the permeation trajectories of cations and anions, resulting in a particularly effective permeation.

We previously reported that millimolar concentrations of LaCl$_3$ added to a symmetric KCl solution also yield a current reduction in OmpF channel [1]. Here we use planar bilayer electrophysiology to understand several characteristic features of the partial channel blocking induced by lanthanum ion: 1) La$^{3+}$ cations reduce the current only when they are added at one side of the membrane (the side of protein addition); 2) The reduction in channel conductance only appears for positive applied potentials; 3) The current inhibition is significantly dependent on the concentration of the supporting electrolyte, KCl; 4) These three effects become apparent with tiny quantities of La$^{3+}$ ions.

We demonstrate that a competitive interaction between La$^{3+}$ and K$^+$ ions is compatible with the asymmetric inhibition. In addition, we analyze the voltage dependence of the current reduction to obtain information about the asymmetry of the blocking. Finally, we show that the substitution of several residues located at the OmpF central constriction can either enhance or inhibit the lanthanum block, pointing out that the interaction between La$^{3+}$ ions and the OmpF channel is likely to occur in the vicinity of the mutated residues.

Pharmacological modulation of cardiac inward rectifier channels. A new challenge.


Cardiac inward rectifier current (I_{K1}) plays a crucial role in the control of the resting membrane potential and of the duration of the final phase of the action potential. Thus, I_{K1} is critical for determining cardiac excitability and refractoriness. Human cardiac ventricular I_{K1} is mainly carried by Kir2.1 homotetramers, while the relative importance of Kir2.2 and 2.3 subunits seems to be greater at the atrial level. I_{K1} inward rectification is due to the voltage-dependent block caused by intracellular Mg^{2+} and polyamines interacting with acidic residues lining the transmembrane and cytoplasmic pore.

Kir channels have been implicated in the perpetuation of reentrant tachyarrhythmias in humans. Indeed, mutations that increase I_{K1} lead to short QT syndrome type 3 and severe ventricular arrhythmias. Additionally, atrial fibrillation, the most common arrhythmia, produces an I_{K1} up-regulation, which is considered critical for the stabilization of the arrhythmia. Therefore, I_{K1} inhibition could offer a potentially useful antiarrhythmic strategy against fibrillatory arrhythmias. Moreover, since Kir2.2 and Kir2.3 seem to be the main atrial Kir2.x isoforms, the selective modulation of Kir2.2-2.3 channels as an atrial selective target for the design of new antiarrhythmic agents could be proposed.

Unfortunately, pharmacology of Kir2.x channels remains elusive. Here, we analyzed the effects of flecainide and propafenone, two class Ic antiarrhythmic drugs, very effective for the acute cardioversion of AF that exhibit ventricular proarrhythmic effects which limit their use. Both drugs, at therapeutic concentrations, selectively increase the current generated by Kir2.1 homotetramers by decreasing channel affinity for polyamines, an effect that decreases inward rectification. Increasing effects are mediated by drug binding to Cys311 and could contribute to their ventricular proarrhythmic effects.

At higher concentrations propafenone, but not flecainide, inhibits the current generated by Kir2.x channels by promoting the appearance of subconductance levels in the channel gating. Propafenone blocks Kir2.x channels by binding in its cationic form to a site located at the interface between subunits in the cytoplasmic domain. Incorporation of positive charges at the level of Arg228 and Arg260 (Kir2.1 residues conserved in Kir2.2 and 2.3) decreases K^+ concentration on the cytoplasmic pore, which in turn, does not supply enough K^+ ions to the transmembrane region avoiding the fully coordination of K^+ ions within the selectivity filter and decreasing the affinity of the channel for PIP_2.

Our results could contribute to take a step forward in our knowledge of the pharmacological modulation of Kir2.x channels and its putative therapeutic utility.

Insights into itch associated to chronic liver disease

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Persistent pruritus is a common disabling dermatologic symptom associated with different etiologic factors. These include primary skin conditions, as well as neuropathic, psychogenic or systemic disorders like chronic liver disease. Defective clearance of potential pruritogenic substances that activate itch-specific neurons innervating the skin is thought to contribute to cholestatic pruritus. However, because the underlying disease-specific pruritogens and itch-specific neuronal pathways and mechanism(s) are unknown, symptomatic therapeutic intervention often leads to no or only limited success. In the current study, we aimed to first validate rats with bile duct ligation (BDL) as a model for hepatic pruritus, and then to evaluate the contribution of inflammation, peripheral neuronal sensitization, and specific signaling pathways and subpopulations of itch-responsive neurons to scratching behavior and thermal hypersensitivity. Chronic BDL rats displayed enhanced scratching behavior and thermal hyperalgesia indicative of peripheral neuroinflammation. BDL-induced itch and hypersensitivity involved a minor contribution of histaminergic-serotonergic receptors, but significant activation of PAR2 receptors, prostaglandin PGE2 formation and potentiation of TRPV1 channel activity. The sensitization of DRG nociceptors in BDL rats was associated with increased surface expression of PAR2 and TRPV1 proteins and an increase in the number of PAR2- and TRPV1-expressing peptidergic neurons together with a shift of TRPV1 receptor expression to medium-sized DRG neurons. These results suggest that pruritus and hyperalgesia in chronic cholestatic BDL rats are associated with neuroinflammation and involves PAR2-induced TRPV1 sensitization. Thus, pharmacological modulation of PAR2 and/or TRPV1 may be a valuable therapeutic approach for patients with chronic liver pruritus refractory to conventional treatments.

Supported by MICINN (BFU2009-08346 to AF-M; SAF2008-00062 to VF and SAF2007-63193 to RP-C), the Consolider-Ingenio 2010 program (CSD2008-00005 to AF-M, VF and RP-C), and la Generalitat Valenciana (Prometeo-2010-046 to AF-M, AP-092/09, Prometeo-2009-027 and ACOMP2010-220 to VF).
Targeting TRP Channels for the Relief of Cold Discomfort and Cold Pain

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TRPM8 and TRPA1 are two cold-activated channels of the Transient Receptor Potential family expressed in different subpopulations of mammalian primary sensory neurons. In recombinant systems, TRPA1 has a much lower temperature threshold than TRPM8, suggesting a specific role in cold pain. In contrast, TRPM8 is clearly involved in sensing cool temperatures but its role in physiological or pathological cold pain is debated. We found that differential expression of Kv1, shaker-like, potassium channels in some TRPM8-expressing cold receptors acts as an excitability brake, expanding the operational range of these neurons into the noxious range. Moreover, paw injection of -dendrotoxin, a selective Kv1 blocker, activated cold-sensitive neurons and caused cold hyperalgesia in mice, suggesting an important function of these potassium channels in cold sensing. We discovered posttranslational mechanisms involved in the modulation of TRPM8 sensitivity to temperature. The localization of TRPM8 within membrane lipid rafts inhibits cold sensitivity. In contrast, the N-glycosylation of TRPM8 channels facilitates their gating by cold and cooling agonists (e.g. menthol), shifting the threshold to warmer temperatures.

Recently, we examined the potential role of TRPM8 and TRPA1 in cold hypersensitivity secondary to oxaliplatin administration, a platinum based chemotherapeutic agent used in the treatment of colorectal cancer. We developed an in vivo mouse model of oxaliplatin-induced cold hypersensitivity. In parallel, we examined the effect of oxaliplatin on TRPM8 and TRPA1 channels expressed heterologously. We found that TRPA1, but not TRPM8, can be activated dose-dependently by oxaliplatin. A single i.p. oxaliplatin injection can produce long-lasting cold hyperalgesia. This cold hypersensitivity was not observed in TRPA1 ko mice and was suppressed by AP-18, a selective TRPA1 antagonist. Interestingly, basal detection of noxious cold was unaffected in TRPA1 ko animals. These results suggest differential roles of TRPM8 and TRPA1 in normal and pathological cold pain, opening new avenues for their selective treatment.

Supported by SAF2010-14990
The neuronal serum- and glucocorticoid-regulated kinase 1.1 reduces neuronal excitability and protects against seizures through upregulation of the M-current


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The M-current formed by tetramerization of Kv7.2 and Kv7.3 subunits is a neuronal voltage gated K⁺ conductance that controls resting membrane potential and cell excitability. In *Xenopus laevis* oocytes, an increase in Kv7.2/3 function by the serum- and glucocorticoid-regulated kinase 1 (SGK1) has been reported (Schuetz et al., 2008). We now show that the neuronal isoform of this kinase (SGK1.1), with distinct subcellular localization and modulation, up-regulates the Kv7.2/3 current in *Xenopus* oocytes and mammalian HEK293 cells. In contrast to the ubiquitously expressed SGK1, the neuronal isoform SGK1.1 interacts with PIP₂ and is distinctly localized to the plasma membrane (Arteaga et al., 2008). An SGK1.1 mutant with disrupted PIP₂ binding sites produced no effect on Kv7.2/3 current amplitude. SGK1.1 failed to modify the voltage dependence of activation and did not change activation or deactivation kinetics of Kv7.2/3 channels. These results suggest that the kinase increases channel membrane abundance, which was confirmed with flow cytometry assays. To evaluate the effect of the kinase in neuronal excitability we generated a transgenic mouse (Tg.sgk) expressing a constitutively active form of SGK1.1 (S515D). Superior cervical ganglion (SCG) neurons isolated from Tg.sgk mice showed a significant increase in M-current levels, paralleled by reduced excitability and more negative resting potentials. SGK1.1 effect on M-current in Tg.sgk-SCG neurons was abrogated after muscarinic receptor activation. Transgenic mice with increased SGK1.1 activity also showed diminished sensitivity to kainic acid-induced seizures. Altogether, our results unveil a novel role of SGK1.1 as a physiological regulator of the M-current and neuronal excitability.
Analysis of a MLC1 KO Mouse Models Provide New Insights in the Pathophysiology of the Leukodystrophy MLC: Chloride Channels are not Working Properly

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Cell volume regulation is pivotal to ensure normal brain function. Its alteration can represent a serious challenge for neuronal survival due to space constrictions within the skull. Thus, brain edema is a major problem in neurology, leading to death in most cases, and it is caused by many defects such as stroke or brain cancer, among others. Our research approaches the study of cell volume regulation in the context of the human genetic disease Megalencephalic Leukoencephalopathy with subcortical Cysts (MLC), as a working model to study brain ionic transport pathophysiology. MLC brains are affected by chronic white matter oedema suggesting a disruption in water and ion homeostasis in astrocytes, which in turn may alter their cell volume regulation abilities. MLC pathology was shown to be primarily caused by a defect in a highly conserved oligomeric plasma membrane protein named MLC1. MLC1 is mostly expressed in astroglial processes and presents low homology to ion channels. However, both the pathophysiological mechanism of MLC disease as well as MLC1 function remained unknown until today, although MLC1 has been related with the activation of volume-regulated chloride channels. Recently, we have identified the second gene responsible for MLC pathology (i.e., GLIALCAM) (1) and described its biochemical role as a MLC1 beta subunit (2). Moreover, we have shown that GlialCAM protein functions as an accessory subunit of the chloride channel ClC-2 (Jeworutzki \textit{et al.}, Neuron (2012)). In this meeting, we will provide new studies with a KO model of MLC1, indicating that dysfunction of chloride channels is a common physiopathological mechanism in MLC disease.

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Hydrophobic Pulmonary Surfactant Proteins SP-B and SP-C Induce Pore Formation in Planar Lipid Membranes: Evidence for Proteolipid Pores

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Pulmonary surfactant is a complex mixture of lipids and specific surfactant proteins, including the hydrophobic proteins SP-B and SP-C, in charge of stabilizing the respiratory surface of mammalian lungs. The combined action of both proteins is responsible for the proper structure and dynamics of membrane arrays in the pulmonary surfactant network that covers the respiratory surface. In the present study we explore the possibility that proteins SP-B and SP-C induce the permeabilization of phospholipid membranes via pore formation. To this end, electrophysiological measurements have been carried out in planar lipid membranes prepared with different lipid/protein mixtures. Our main result is that channel-like structures are detected in the presence of SP-B, SP-C or the native mixture of both proteins. Current traces show a high variety of conductance states (from pS to nS) that are dependent both on the lipid composition and the applied potential. We also show that the type of host lipid crucially determines the ionic selectivity of the observed pores: the anionic selectivity observed in zwitterionic membranes is inverted to cationic selectivity in the presence of negatively-charged lipids. All those results suggest that SP-B and SP-C proteins promote the formation of proteolipid channels in which lipid molecules are functionally involved. We propose that proteolipidic membrane-permeabilizing structures may have an important role to tune ionic and lipidic flows through the pulmonary surfactant membrane network at the alveolar surfaces.

We acknowledge funding from the Spanish Ministry of Economy and Competitivity (BFU2010-11538-E, BIO2012-30733, FIS2010-19810, CSD2007-0010), Fundació Caixa Castelló-Bancaixa (P1-1A2009-13), Generalitat Valenciana (Prometeo 2012/069) and Madrid Regional Government ((S2009MAT-1507), and a fellowship from the Spanish Society for Pneumology and Thoracic Surgery (SEPAR 956/2010).
POSTER ABSTRACTS
Mammalian KCNQ genes encode five Kv7 potassium channel subunits (Kv7.1-Kv7.5). Kv7.2 and Kv7.3 are expressed in the nervous system, being the principal molecular components of the slow voltage gated M-channel, which exert a strong control in neuronal excitability. Calmodulin (CaM) binds to two sites named helix A and B within the intracellular C-terminus, mediates inhibition of Kv7.2 channels and is required for the channels to exit the endoplasmic reticulum. The molecular details of how CaM trigger channel trafficking or the reduction of M-current are unknown. The aim of this study was to explore the molecular events within CaM elicited by Ca2+ using two complementary approaches. In one, we have performed a fluorimetric assay using dansylated calmodulin (D-CaM) to characterize the interaction of individual lobes to the Kv7.2 CaM binding site. The association of the Kv7.2 with CaM was also explored using NMR spectroscopy, employing 15N-labeled CaM as a reporter. Our data show interdependency of the N- and C-lobes in the interaction and suggest that Ca2+ causes the contacts with CaM to pivot between EF-1 in the N-lobe (whose interactions is dominated by helix B) and EF-4 in the C-lobe (where the predominant interaction is with helix A). In addition, Ca2+ makes CaM binding to the channel more difficult, and, reciprocally, the channel weakens CaM binding to Ca2+. 
Subcellular localization of $\alpha$ and auxiliary subunits of BK channels

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Large-conductance $\text{Ca}^{2+}$- and voltage-activated $\text{K}^+$ (BK) channels play an important role in neuronal excitation. $\text{K}_{\text{Ca}}$ channel is a tetrameric assembly of $\alpha$-subunits ($\text{BK}_\alpha$) which are all functional per se. However, in vertebrates, the majority of $\text{BK}_{\text{Ca}}$ channels are associated with auxiliary $\beta$-subunits. BK channels are widely expressed throughout the nervous system but little is known about the $\alpha$-$\beta$ stoichiometry and their regional and subcellular distribution in some regions as the cerebellum. We have focused our study in the $\alpha$ structural and $\beta_3$ auxiliary subunits to determine both expression patterns and their possible overlapping. In the present study, we used histoblotting and pre-embedding electron microscopy techniques to reveal the regional distribution and subcellular localization of BK channels in the central nervous system and in the cerebellum, respectively. We found that both subunits are widely expressed in the central nervous system with high levels of expression in some regions such as hilus in the hippocampus and molecular layer in the cerebellum. Electron microscopy confirmed localization of BK channels at postsynaptic levels in the molecular layer of the cerebellum and, to a lesser extent, at presynaptic levels. The similar patterns of subcellular localization of $\alpha$ and $\beta_3$ subunits in axonal as well as dendritic membranes of glutamatergic synapses in the cerebellum suggest that $\beta_3$ subunit is intimately associated with $\alpha$ subunit in this region.

Supported by MICINN grant (BFU-2009-08404 and CSD2008-00005).
Objective: Vascular smooth muscle contracts in response to an increase in intracellular Ca\textsuperscript{2+} owing to Ca\textsuperscript{2+} release from sarcoplasmic reticulum (SR) and Ca\textsuperscript{2+} entry through voltage dependent and independent ion channels. Voltage-dependent Ca\textsubscript{V}1.2 L-type Ca\textsuperscript{2+} channels (LTCC) are considered the main route for calcium entry in vascular smooth muscle cells (VSMC). However, several studies have determined the relevant role of store-operated Ca\textsuperscript{2+} channels (SOCC) in vascular tone regulation (1). The aim of this study was to characterize the communication between SOCC and LTCC and their role in the agonist-induced vasoconstriction.

Materials and Methods: We used wild type and smooth muscle-selective conditional Ca\textsubscript{V}1.2 knockout mice (2) to study arterial rings contractility as well as intracellular Ca\textsuperscript{2+} mobilization and membrane potential changes in isolated VSMC.

Results: We found that agonist as serotonin induced aorta vasoconstriction was sensitive to LTCC and SOCC inhibitors indicating the participation of both conductances in vessels contraction. Moreover, vasoconstriction induced by agonist or by thapsigargin, which selectively activate SOCC, was attenuated in arterial rings from Ca\textsubscript{V}1.2 knockout mice compared to wild type. We observed in isolated VSMC that thapsigargin evoked a prominent cytosolic Ca\textsuperscript{2+} increase that was sensitive to nifedipine demonstrating that SOCC activation promotes LTCC opening. Furthermore, in current clamp configuration, thapsigargin induced the depolarization of wild type VSMC which was significantly lower in Ca\textsubscript{V}1.2 knockout VSMC confirming that SOCC activated pathway involves a depolarization and further LTCC opening.

Conclusions: These data suggest a functional interaction and co-activation of SOCC and LTCC in VSMC. LTCC activation during agonist-induced vasoconstriction seems triggered by store-operated Ca\textsuperscript{2+} entry through SOCC.

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Supported by Spanish Ministry of Innovation and Science (BFU2010-21043-CO2-2); by Andalucía Government (P10-CVI-6095).
Progesterone Modulates Ciliary Activity in Mouse Oviduct Ciliated Epithelial Cells

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Ciliary activity in the oviduct is responsible for ovum pickup as well as tubal fluid distribution, which supports gamete maturation and transport. Ciliary beat frequency (CBF) is modulated by different cellular signals, being Ca²⁺ the main CBF activator, and during the oestrous cycle by the female sex hormones progesterone (PG) and oestrogen. Direct analysis of the impact of PG on oviduct CBF has produced conflicting results, probably due to differences in the species, sample preparation, concentrations and method of CBF recording used. Although, most agree that long exposure to PG reduces CBF.

Here we show that acute application of progesterone increases cytosolic Ca²⁺ and CBF in mouse oviduct ciliated epithelial cells. Both Ca²⁺ signals and CBF increase could not be prevented by RU-486, the classical progesterone receptor antagonist but were attenuated by U73122, a Phospholipase C (PLC) inhibitor, implying that progesterone activated CBF through a signaling pathway involving PLC activation and Ca²⁺ mobilization. Amongst the potential candidate proteins that could activate PLC and generate Ca²⁺ signals, there are membrane progesterone receptors (mPRs), the progesterone membrane receptor component 1 and 2 (PGRMC1 and PGRMC2) and G-protein coupled receptors (GPCRs). We found that baclofen, an agonist of the GPCR GABA-B receptors, also increased CBF and Ca²⁺ signals and that TRPV4 channels participate in the progesterone-induced cytosolic Ca²⁺ signals.

In conclusion, progesterone-induced activation of CBF in mouse ciliated epithelial cells may involve the activation of a molecular macro-complex composed of GABA-B receptors and TRPV4 channels leading to PLC activation and cytosolic Ca²⁺ mobilization.

Supported by the Spanish Ministry of Science and Innovation (SAF2009-09848 and SAF2012-38140); Fondo de Investigación Sanitaria (Red HERACLES RD12/0042/0014); FEDER Funds; Generalitat de Catalunya (SGR05-266); and Fundació Marató de TV3 (080430). C.J is the recipient of a Juan de la Cierva Fellowship and M.A.V. is the recipient of an ICREA Academia Award.
Subcellular association between GIRK Channels and the RGS7 and Gβ5 Proteins

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The GIRK channels mediated the inhibitory postsynaptic effect of neurotransmitters and abuse drugs. These channels are characterized by their insensitivity to voltage but activation through G proteins. G proteins mediated signaling is one for the most widely used trans-membrane signaling mechanisms in mammalian organism. There are a number of factors participating in the G protein-signaling system to modulate functional association of GIRK channels and their cognate G protein-coupled receptors (GPCR). Members of the regulator of G protein signaling (RGS) protein family are important intracellular factors acting in the activation and inactivation of GIRK channels. RGS proteins serve as negative regulators of GPCR signaling by stimulating GTP hydrolysis on the Gα subunits to promote their inactivation. There is now compelling evidence that all member of the R7 RGS protein family exists as complexes with type 5 G protein β subunit (Gβ5). However, little information is available on their cellular and subcellular localization. We have previously shown that GIRK2 interact with GIRK1 and GABA_B1 receptors. Using expression systems and co-immunoprecipitation approaches, we show that GIRK2 interact with RGS7 and Gβ5 in HEK 293 FT cells. Using high-resolution immunoelectron microscopic techniques, we observed that RGS7 and Gβ5 were located in CA1 pyramidal cells at postsynaptic and presynaptic sites. The use of quantitative approaches at the EM level have shown that RGS7 and Gβ5 immunoparticles were detected both at intracellular sites (56% for RGS7 and 75% for Gβ5) and along the plasma membrane (44% for RGS7 and 25% for Gβ5). Of the immunoparticles detected along the plasma membrane in the stratum radiatum of the CA1 region, most were detected at postsynaptic sites (5%) in axon terminals, likely Schaffer’s collaterals, establishing excitatory synapses with spines. In dendritic spines of pyramidal cells, both RGS7 and Gβ5 showed the same distribution relative to the glutamate release site, also very similar to the distribution described for GABA_B receptors and GIRK channels in the same GABA_B compartment. Our molecular, cellular and subcellular studies suggest that RGS7 and Gβ5 are forming macromolecular complexes with GIRK and GABA_B, and suggest that RGS7-Gβ5 complexes may be critical in the regulation and activation of GIRK channels.

Supported by MICINN grant (BFU-2009-08404 and CSD2008-00005).
Role of Connexin-36 Hemichannels in Insulin Secretion


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Glucose-stimulated insulin secretion from β-cells is mediated by an elevated ATP/ADP ratio following glucose metabolism, subsequent ATP-sensitive K+ channel closure, membrane depolarization, Ca2+ influx and [Ca2+]i oscillations, and insulin granule exocytosis. Optimum insulin secretion also requires the synchronization of β-cells within pancreatic islets via connexin-36 (Cx36) gap junction channels which generates the coordination of [Ca2+]i and a pulsatile insulin secretion. However, the role played by Cx36 hemichannels (i.e., the precursors of intercellular channels) which can exchange ions, second messengers and metabolites between the cell interior and interstitial space is still to be elucidated. We hypothesize that mice lacking Cx36 (Cx36−/−) would show defects on glucose homeostasis by disruption of not only the function of intercellular channels but also of hemichannels.

Our results show that Cx36−/− mice are slightly glucose intolerant despite normal insulin sensitivity and they show reduced fasting plasma glucose and insulin levels. Some of the changes found in isolated islets are compatible with these results. Insulin release at 5 mM glucose (G5) is lower in perifused Cx36−/− than in wild type islets. In addition, the response to 20 mM glucose (G20) is impaired showing a significant larger reduction of the second and sustained phase of insulin release than the initial phase. Compared with wild controls, Cx36−/− islets had higher ATP content at G5 but a normal ATP/ADP ratio while both values were similarly increased by G20. Depolarization with 70 mM KCl at G5 decreased ATP content in wild controls but not in Cx36−/− islets. This ATP depletion was reversed by extracellular ATP (5 mM) that stimulated a second and sustained phase of insulin secretion after the transient peak triggered by KCl. Taken together these results clearly indicate that ATP is released and taken up throughout Cx36 hemichannels, suggesting that they can be important for the regulation of insulin secretion.

Supported by grants of the Spanish Ministry of Science and Technology (SAF-2009/1164, SAF2009/1267, and Consolider CSD2008-00005) and the Community of Madrid (Neurotec-P2010/BMD-2460)
Kv7.1/Kv7.5 Heteromeric Channels In Cardiovascular Smooth Muscle

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Aims

Voltage-dependent K⁺ channels (Kv) from the Kv7 family are implicated in modulating vascular smooth-muscle tone. Specifically, Kv7.1, Kv7.4 and Kv7.5 are expressed in blood vessels and contribute to cardiovascular physiology. While Kv7 channel blockers trigger muscle contractions, Kv7 channel activators act as vasorelaxants. Although Kv7.1 is highly expressed in many vessels, Kv7.1 blockers fail to modulate smooth-muscle reactivity. For this reason, the role of the Kv7.1 channel is under intense investigation. Neuronal Kv7 channels form heterotetramers, whereas Kv7.1 association is notably restricted. We wanted to analyse the possibility of Kv7.1 and Kv7.5 forming heteromeric channels.

Materials and Methods

We performed electrophysiological studies in Xenopus oocytes, and confocal microscopy and lipid raft isolation in HEK-293 cells and myoblasts.

Results

In this study, we demonstrate that Kv7.1 and Kv7.5 can form functional heterotetrameric channels. The hybrid Kv7.1/Kv7.5 structures have a characteristic phenotype. Similar to Kv7.1, Kv7.1/Kv7.5 heteromeric channels are highly retained at the endoplasmic reticulum. However, studies in heart, myoblasts and HEK-293 cells demonstrate that unlike Kv7.1, the presence of Kv7.5 stimulates release of Kv7.1/Kv7.5 oligomers out of lipid rafts.

Conclusions

Because the lipid raft localization of ion channels is crucial for cardiovascular physiology, we propose that the formation of Kv7.1/Kv7.5 heteromeric channels provides efficient spatial and temporal regulation of smooth-muscle cell function. Our results shed light on the debate regarding the relative contribution of Kv7 channels to vasoconstriction and hypertension.

Supported by BFU2011-23268 and CSD2008-00005 (MINECO, Spain).
ORMDL3 Affects the Calcium Dependent Inactivation of I_{CRAC}

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Objectives: The Store Operated Calcium Entry (SOCE), which is essential for T lymphocyte activation, is triggered by the depletion of the Endoplasmic reticulum (ER) Ca^{++} stores. Here we report that the ER located ORMDL3 (product of the ORMDL3 gene associated with many inflammatory diseases) modulates SOCE response. In T cells, the main current for SOCE is Ca^{++} release-activated Ca^{++} currents (I_{CRAC}). Therefore, we recorded endogenous whole-cell I_{CRAC} in Jurkat T cells overexpressing or downregulating ORMDL3 in order to identify the mechanism behind ORMDL3 modulation of I_{CRAC}.

Results: We measured I_{CRAC} using the slow Ca^{++} chelator EGTA (10mM) to see the normal development of the current. Our results showed that cells overexpressing ORMDL3-WT presented a decrease in current density and a delay in the development of the current compared to control cells. Besides, downregulation of ORMDL3 produced an increase in current density and a faster development of I_{CRAC} compared with control siRNA. Interestingly, the ORMDL3 effect on CRAC channels was completely abolished when the fast Ca^{++} chelator BAPTA was used instead of EGTA. Altogether, these experiments suggested that ORMDL3 affects the Calcium Dependent Inactivation (CDI) of CRAC channels. Supporting this idea, we performed experiments in the presence of Thapsigargin (a SERCA pump inhibitor), in the absence of external calcium, and with a weak intracellular Ca^{++} buffering solution. After two minutes we added Ca^{++} to the bath to completely develop ICRAC, revealing the CDI of CRAC channels. Under these conditions, ORMDL3 overexpressing cells inactivate I_{CRAC} faster than control cells, an effect that was abolished when using strong calcium buffering conditions.

Conclusions: Hence, we demonstrate that ORMDL3 levels negatively modulate I_{CRAC} by affecting the Calcium Dependent Inactivation of the CRAC channels.

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This work was supported by Spanish Ministry of Science and Innovation (SAF2009-09848; SAF2010-16725, SAF2012-38140), Fondo de Investigación Sanitaria (Red HERACLES RD12/0042/0014), FEDER Funds; Generalitat de Catalunya (SGR05-266) and Fundació la Marató de TV3 (080430).
Novel Mechanism for ORMDL3 to Regulate SOCE in a T Cell Model


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ORMDL3 gene has been related by Genome Wide Association Studies (GWAS) to different proinflammatory and autoimmune diseases like: Asthma, type 1 diabetes, ulcerative colitis, Crohn’s disease and ankylosing spondilitis. The product of this gene, ORMDL3 protein, inhibits Sarco Endoplasmic Calcium pump 2b (SERCA2B) and affects cellular calcium homeostasis. However, our data show that ORMDL3 expression regulates Store Operated Calcium Entry (SOCE) independently of SERCA activity (1). Under T cell activation mitochondria surround T cell receptor and maintain SOCE by clearing calcium from Orai’s pore. Here we want to address whether ORMDL3 interacts with mitochondria and how this might affect SOCE in lymphocytes.

The Jurkat T cell line was heterogously transfected with ORMDL3 WT and different mutants to delimitate functional domains. Downregulation was achieved by transfecting with ORMDL3 siRNA. Store Operated Calcium Entry (SOCE) was evaluated by store depletion with ciclopiazonic acid (CPA) or with CD3 crosslinking. ORMDL3 overexpression decreased SOCE compared to ΔN mutant and this decrease could be reverted or mimicked by chemical interventions into mitochondria. Besides, ORMDL3 was present in ER-mithondria interacting sites. To further evaluate mitochondrial function, Rhodamine 5N-AM mitochondrial calcium probe or a ratiometric mitochondrial pericam probe (mtpericam) were used. Live calcium imaging using both approaches revealed that ORMDL3 dramatically decreases mitochondrial calcium buffering capacity. Mutant studies also suggested that the amino terminal part of ORMDL3 is necessary to trigger this effect.

This work reveals that ORMDL3 decreases mitochondrial calcium buffering capacity thereby affecting T cell calcium signalling. We also define the amino terminal end of this protein as the domain responsible for this effect.

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This work was supported by Spanish Ministry of Science and Innovation (SAF2009-09848; SAF2010-16725, SAF2012-38140), Fondo de Investigación Sanitaria (Red HERACLES RD12/0042/0014), FEDER Funds; Generalitat de Catalunya (SGR05-266) and Fundació la Marató de TV3 (080430).
Characterisation of the functional expression of TRESK (K$_2$P18.1) in *Saccharomyces cerevisiae*.

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TRESK (K$_2$P18.1) is a two-pore domain potassium channel that is highly expressed in the peripheral nervous system, particularly in sensory neurons and is implicated in control of neuronal excitability. It represents a potential therapeutic target for treatment of migraine and neuropathic pain.

The aim of this study was to characterise functional expression of K$_2$P18.1 in potassium-transport deficient strains of *Saccharomyces cerevisiae*, with a view to developing a model system for trafficking, pharmacological and structure-function studies of K$_2$P18.1.

A vector expressing rat K$_2$P18.1 under the control of a constitutive yeast PGK promoter was constructed (HA-rK$_2$P18.1). A HA tag preceded by a yeast Kozak sequence was engineered onto the channel N-terminus to enable monitoring of protein expression. HA-rK$_2$P18.1 was transformed into two potassium-transport deficient yeast strains; WΔ3, which lacks the transporters required for high-affinity potassium uptake (TRK1 and TRK2), and MAB-2d, which, in addition to lacking TRK1 and TRK2, carries genetic deletions of the potassium efflux transporters ENA1 and NHA1. Both strains require media supplementation with 100mM KCl to permit growth. We hypothesised that functional expression of K$_2$P18.1 would permit these strains to grow on media not supplemented with KCl by virtue of the channel allowing sufficient potassium entry for growth. Furthermore, a predicted consequence of K$_2$P18.1 channel expression in MAB-2d would be growth inhibition upon exposure to high potassium concentrations (600mM) due to a lack of the potassium efflux systems required to eliminate excess potassium entry via a functional K$_2$P18.1.

Our preliminary results demonstrate that protein expression of HA-rK$_2$P18.1 could be observed by Western blot in both strains. It appears that expression of HA-rK$_2$P18.1 is not sufficient to permit growth of either strain in low potassium medium. However, robust inhibition of growth in the presence of 600mM KCl was observed in the MAB-2d strain when HA-rK$_2$P18.1 was expressed, suggesting that rat K$_2$P18.1 is functional in *Saccharomyces cerevisiae*.

Supported by grants from and Ministerio de Sanidad of Spain: FIS PI11/01601 and by RETIC RD07/0062/0006 and 2009SGR869, Generalitat de Catalunya. JPG is supported by a Ramón y Cajal fellowship (RYC-2011-08589).
Studying the Mechanisms Involved in the Attenuation of Polyamine Block of AMPARs by Auxiliary Subunits.

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AMPA receptors (AMPAR) mediate most of fast excitatory neurotransmission in the brain. A subclass of AMPARs (calcium permeable; CP-AMPARs) is subject to modulation by intracellular polyamines, which block them in a voltage dependent manner. This block limits the calcium entry in neurons under strong depolarization circumstances – for example during sustained activity or pathological conditions as ischaemic processes. AMPAR function is controlled by auxiliary subunits (transmembrane AMPA receptor regulatory proteins; TARPs), which affect the trafficking and gating properties of AMPARs. One of the numerous effects of TARPs over AMPARs is the attenuation of polyamine (PA) block of CP-AMPARs that is less strong in the presence of TARPs (Soto et al., 2007; Soto et al., 2009) thus increasing the calcium influx through CP-AMPARs. However, the molecular mechanisms or the TARP domains involved in such attenuation remain a mystery. The goal of this work is to characterize the domains/aminocids that are important in that attenuation. For this study we have used the patch-clamp technique to record glutamate-activated currents of tsA201 cell line transfected with expression vectors codifying for CP-AMPAR subunits and the prototypical TARP, γ-2. To study key aminoacids or domains of γ-2 involved in PA block attenuation, we have done site-directed mutagenesis onto plasmids that codify for γ-2 in order to change specific residues of the carboxyl-terminal domain. We have created as well a version of γ-2 without the whole carboxyl-terminal domain to investigate the role of it in PA block attenuation. Our results demonstrate that the C-terminal domain of γ-2 is partially involved in the attenuation of PA block. However our data seems to indicate that a different part of the molecule interacting with the AMPAR pore-forming subunits might be involved in the effect.

References:


Store-operated Ca$^{2+}$ Currents in Vascular Smooth Muscle and Colonic Cells Studied by Planar patch-clamp

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Ca$^{2+}$ is a second messenger involved in many cellular processes including cell proliferation. A critical pathway in this regard are the store-operated Ca$^{2+}$ channels activated by Ca$^{2+}$ release from intracellular stores and modulated by mitochondria. The molecular identity, biophysical properties and physiological functions of store-operated currents (SOCs) in different cell types are still not clear. Patch-clamp electrophysiology is an excellent technique for studying ion channels, but the extremely low single conductance (~fS) of SOCs hampers their characterization. The present work has focused on electrical recording of SOCs among different cells using an automated patch-clamp device.

A Port-a-patch planar patch-clamp system (Nanion Technologies) was used for high resolution electrophysiological recordings (in voltage-clamp whole-cell configuration). Using borosilicate-glass chips, we were able to achieve “giga-seals” (>1GΩ) and recorded suspended cells including pituitary, vascular smooth muscle and colonic cells. SOCs were measured by applying voltage ramps (-100 to +100 mV) from a holding potential of 0 mV. For activation, intracellular Ca$^{2+}$ stores were depleted by chelating cytosolic Ca$^{2+}$, intracellular thapsigargin and IP$_3$ and external thapsigargin.

SOCs were found in all cell lines studied (amplitude 1-10 pA/pF). $I/V$ relationships were characterized by small inward current amplitude, voltage-independent activation, inward rectification, and reversal potential in positive voltages. High intracellular EGTA decreased slow inactivation and amplitude was reduced by 2APB.

In all cell lines examined SOCs could be accurately examined with planar patch-clamp. The recordings obtained allow us preliminary biophysical and pharmacological characterization of SOCs that may play important roles in these cells, particularly in cell proliferation.

Supported by DIGICYT (BFU2009-08967) and by European Social Fund and Conserjería de Educación, Junta de Castilla y León (VA270A11-2).
TRCP1 is Linked to Group I Metabotropic Glutamate Receptors Pathway in Auditory Midbrain Neurons

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Group I metabotropic glutamate receptors (mGluRs) are widely expressed in the central nervous system playing multiple roles in normal and pathological situations. Recently we have demonstrated that during auditory development, group I mGluR expression and activity are down-regulated (1). In the present work, we have studied the signaling routes triggered by group I mGluR activation in auditory brainstem neurons, namely in the central nucleus of the inferior colliculus (CIC). We have also characterized the expression of possible molecules candidates to be activated through this pathway.

Optical imaging of the fluorescent Ca2+-sensitive dye Fura-2, in postnatal midbrain slices, was used. Application of DHPG, a specific agonist of group I mGluRs, elicited noticeable [Ca2+]i responses in most of the CIC neurons. They consisted of an initial Ca2+ peak followed by a plateau phase. Thapsigargin blockade of intracellular Ca2+ release demonstrated that store depletion was necessary for the initial [Ca2+]i peak. A subsequent extracellular Ca2+ entry, insensitive to voltage-gated calcium channel blockers, was present during the plateau phase. Such influx was sensitive to 2-APB, a blocker of the transient receptor potential (TRP) and store-operated calcium (SOC) channels. To establish the molecular components involved in this current, immunohistochemistry against TRPC1, or Stim1/Orai1 proteins, known components of the SOC machinery, was performed. Our results show that TRPC1 is widely expressed in CIC neurons, whereas neither Stim1 nor Orai1 labeling was found in this region. Confocal double immunofluorescence was then used to study the cell type and subcellular compartment where TRPC1 was present. TRPC1 was expressed in a subpopulation of CIC neurons positive to parvalbumin and lacking in GFAP-positive glial cells. Furthermore, this channel was widely distributed in MAP-2 immunoreactive somata and dendrites and absent in synaptophysin-positive axonic terminals. Our results suggest for the first time that, in auditory brainstem neurons, TRPC1 could act downstream of mGluR signaling pathway.

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Supported by MCINN (BFU2009-13754-C02-01) to JMJ and by Consejería de Educación JCCM (P11109-0056-7896) to JR MG.
Kv1.3 Channel Inactivation is Modulated by Intracellular Calcium

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Objective: Kv1.3 is a ubiquitously expressed voltage-gated K\textsuperscript{+} channel with roles in a variety of physiological processes. This Shaker-related K\textsuperscript{+} conductance is strongly activated by depolarisation and shows marked “C-type” inactivation. A recent study in platelets and megakaryocytes, has demonstrated that Kv1.3 is the exclusive voltage-gated K\textsuperscript{+} channel and the main determinant of the resting membrane potential (McCloskey et al, J Physiol 588.9 (2010)). We have now investigated the regulation of Kv1.3 during activation of GPCRs with the aim of understanding how this conductance may contribute to membrane potential regulation during Ca\textsuperscript{2+} signalling.

Materials and Methods: Simultaneous electrophysiological recordings (whole-cell patch clamp or TEVC) and Ca\textsuperscript{2+} microfluorimetry were performed in megakaryocytes from either wild-type or Kv1.3-deficient mice and different cellular models expressing Kv1.3 (COS-7 cells -gift of S.Grissmer- and *Xenopus* oocytes).

Results: Purinergic P2Y\textsubscript{1} GPCR stimulation (1 µM ADP) profoundly reduced the voltage-dependent inactivation of Kv1.3 channels in mouse megakaryocytes. The current at the end of 3s step depolarisations (-80mV to +60mV) was enhanced by up to 8-fold. Experiments with Kv1.3-deficient megakaryocytes and K\textsuperscript{+} channel blockers showed that this effect did not result from activation of a Ca\textsuperscript{2+}-gated K\textsuperscript{+} conductance. The altered P2Y\textsubscript{1}-evoked Kv1.3 inactivation was mediated by an increase in [Ca\textsuperscript{2+}], without a role for protein kinase C (PKC) as it was blocked by intracellular BAPTA, unaffected by the PKC inhibitor Ro-31-8220 and also induced by application of ionomycin. In current-clamp recordings, Ca\textsuperscript{2+}-dependent alteration of Kv1.3 inactivation hyperpolarised the cell during injection of depolarising current pulses and thus likely contributes to the stable membrane potential observed during activation of P2Y\textsubscript{1}-evoked non-selective cation channels. The Ca\textsuperscript{2+}-dependence of Kv1.3 inactivation was not restricted to megakaryocytes, as similar modulation of inactivation was also observed for murine Kv1.3 channels expressed in COS-7 cells or *Xenopus* oocytes.

Conclusions: Ca\textsuperscript{2+}-dependent reduction of Kv1.3 inactivation represents a novel modulatory mechanism whereby GPCR activation prolongs K\textsuperscript{+} currents and stabilises the membrane potential during Ca\textsuperscript{2+} signalling.

Supported by CSD2008-00005 (MICINN, Spain) and the British Heart Foundation.
α9 nAChRs regulate action potential firing and synaptic transmission at the rat adrenal medulla: role of SK channels

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Adrenomedullary chromaffin cells contribute to the “fight or flight” stress response by releasing catecholamines into the blood stream. The cascade of events that at the membrane level ultimately leads to catecholamine secretion (excitation-secretion coupling) begins when acetylcholine (ACh) released from splanchnic nerve terminals synapsing onto chromaffin cells binds to nicotinic acetylcholine receptors (nAChRs) to rapidly depolarize the chromaffin cell membrane (fast EPSP) and, subsequently, trigger the discharge of Na⁺- and Ca²⁺-dependent action potentials (AP). Interestingly, the chromaffin cell secretory response is a function of both the frequency of firing of splanchnic nerve fibers and of chromaffin cells. Small-conductance Ca²⁺ activated K⁺ (SK) channels are well known to control AP firing by producing a slow afterhyperpolarization, which summates along a train of APs to determine the spike-frequency adaptation phenomenon. While Ca²⁺ entry through voltage-dependent Ca²⁺ channels during APs is commonly accepted to be responsible for SK channel activation, ligand gated ion channels (brain NMDA receptors and cochlear α9 nAChRs) have also been proposed to couple synaptic activity to SK channel activation. Here, we report that apamin-sensitive SK channels contribute to AP frequency adaptation observed in electrically-depolarized chromaffin cells from tissue slices of the rat adrenal gland. Exogenous application of ACh (50 µM, 500 ms) also evoked chromaffin cell depolarization, sustained AP firing, and spike frequency adaptation, which was sensitive to apamin (100 nM) too. Interestingly, α-conotoxin Rg1A (200 nM), a selective blocker of the highly Ca²⁺-permeable α9 nAChRs, mimicked the effect of apamin on AP firing elicited by exogenous ACh. This result suggests that besides directly producing membrane depolarization, nAChRs may also indirectly drive the membrane potential in the opposite direction: hyperpolarization produced by SK channels activated by Ca²⁺ entry though nAChRs. This hypothesis has been tested at the splanchnic nerve-chromaffin cell junction where nAChRs concentrate and can be simultaneously activated by brief pulses of ACh released by focal electrical stimulation of splanchnic nerve terminals. Under voltage-clamp conditions, apamin prolonged the late phase of decay of EPSCs, this effect being attenuated as membrane potential (Vh) approached EK. Likewise, current-clamp recordings showed that blocking SK channels increased the EPSPs width.

It is concluded that postsynaptic nAChRs are functionally coupled to SK channels on chromaffin cells, thus implying a role for SK channels in the modulation of synaptic transmission at the adrenal medulla. Defining their contribution to chromaffin cell response during stress as well as their potential colocalisation with nAChRs at the structural level will be the subject of future investigations.

Supported by SICI (CSD CSD2008-00005) and BFU2011-26253 grants
Mapping of Interactions between the Amino and Carboxy Termini and the Channel Core in Herg K^+ Channels

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The potassium channel encoded by the human ether-a-go-go-related gene (h-ERG) contributes to cardiac repolarization as well as setting the electrical behavior of a variety of cell types, due to its characteristic gating properties. Recent analysis by disulfide bond cross-linking between pairs of engineered cysteines indicated that the intramolecular interactions between the hERG N-terminal tail and the S4-S5 linker are part of an interactive network, in which these regions as well as other cytoplasmic domains dynamically contribute to the modulation of channel gating [1]. Whereas it is widely accepted that the interaction between those two regions acts as a critical determinant of the characteristic slow deactivation of hERG, it has been reported that both the adjacent Per-Arnt-Sim (PAS) domain and the remainder of the N-terminus (the proximal domain) play important roles in the activation and deactivation properties of the channel [2].

In this report we analyze the role of the PAS and the proximal domains in determining the position and controlling the access of the N-terminal tail of hERG to its interaction site with the gating machinery. We checked the impact that structural alterations in these regions of the hERG amino terminus exert on disulfide bridge formation between cysteine residues introduced at positions 3 and 542 of the channel sequence. Our results indicate that the presence of an intact PAS domain is necessary for the interaction between the N-terminal tail and the S4-S5 linker, but maintenance of the hERG long proximal domain is not. The state-dependent formation of a disulfide bridge between cysteine at position 3 and an endogenous cysteine located at position 723 in the carboxy terminal C-linker, indicates that the N-terminal tail of hERG also lies in close proximity to the C-linker structures located at the bottom of helix S6.

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Supported by the Spanish Ministerio de Ciencia e Innovación (MICINN) by grant BFU2009-11262 and a Consolider-Ingenio project (SICI, CDS2008-00005).
Molecular Interactions between the Kv1.3 channel and caveolin

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Background

The voltage-dependent K⁺ channel Kv1.3 participates in many physiological events like proliferation and activation of immunitary cells, membrane repolarization in sensory neurons, vascular smooth muscle tone, insulin resistance and obesity. In most cases, Kv1.3 concentrates in specific membrane microdomains called lipid rafts. These domains act as platforms where signaling pathways converge. Caveolae are a specialized form of lipid rafts with, an omega shaped structure build up by the structural protein caveolin. Evidence indicates that Kv1.3 functionality relays not only on expression but also on localization at the plasma membrane. The importance of this spatial regulation is manifest when an altered Kv1.3 distribution is related with the appearance of disease. Therefore, the mechanisms that govern Kv1.3 function are of considerable interest.

Results

In this study we characterized the relationship between Kv1.3 and caveolins. In addition, we analyzed the presence of putative caveolin-binding domains in Kv1.3 that could explain their interaction. To that end, we performed structure-function studies by using a repertoire of Kv1.3 mutants and stable HEK-293 cell lines with or without caveolins, characterized putative interactions and analyzed membrane dynamics by single molecule tracking.

Conclusions

As Kv1.3 is considered an important pharmacological target for different autoimmune diseases and obesity, it is important to bring light to the Kv1.3 interactions with other partners that can regulate channel surface distribution and function.

Supported by BFU2011-23268 and CSD2008-00005 (MINECO, Spain).
One of the aims of our current research program is to look at changes at the cellular and molecular level in central auditory neurons, with special focus on neurotransmitter receptors, ion channels and transporters after interfering with inputs, in order to shed light on possible reactive/plastic mechanisms to altered auditory activity and experience. Deafness represents a drastic change in auditory experience and by extension in the activity patterns of auditory neurons and circuits, which likely involve plastic adaptations in neuronal signaling molecules and mechanisms.

We are exploring whether deafness (i.e. auditory activity) affects the expression of potassium channels in the inferior colliculus, the core integration center in the auditory midbrain. Towards this goal PCR Arrays containing key neuronal potassium channels genes are being used, on the one hand after experimental deafness subsequent to cochlear ablation in the adult Wistar rat at three different times (1 day, 15 days and 90 days postlesion) and, on the other hand, in relation to hearing onset, that is, comparing differences before (postnatal day 9, P9) and after (P14 and P30) hearing onset in the rat, which takes place between P11 and P12.

In deaf adult rats, there is an extensive down-regulation at one day postlesion, which particularly affects Kv1.4, Kv2.1, Kv11.1, Kv9.3, Kir6.1 and Kir6.2. Fifteen days after the lesion, down-regulation persists although it is less pronounced. Ninety days after the lesion expression levels return to control levels. Around and beyond hearing onset, there is an up-regulation in the expression of potassium channel genes, which extends up to P30. Up-regulation is seen especially in Kv1.1, Kv1.2, Kv3.1, Kv3.3, Kv9.3 and Kir 6.2.

In summary, these findings suggest that the expression of potassium channel genes likely is activity-dependent in the inferior colliculus. This may important roles in adapting central neuronal excitability to ongoing levels of activity under normal and pathological conditions, including deafness.

Supported by MICIIN BFU2009-13754-C02-01 and PEII09-0152-6233 (JCCM).
Single Channel Recording of K2P Channels in Peripheral Ganglia.


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Objectives: The first member of the K2P channel family was cloned in 1996, since then several members have been added to this group and their presence in most of the areas in the central and peripheral nervous system have been described. Our group recently described the presence of functional K2P channels in the superior cervical and nodose ganglia (SCG and NG), demonstrating their role in the maintenance of the resting membrane potential and excitability (Cadaveira-Mosquera et al, 2011; 2012). The goal of the present study is to further investigate the characteristics of these conductances at the single channel level in the SCG and NG.

Materials and methods: Primary cultured neurons from SCG or NG, obtained from mice 30 – 60 days old, were recorded using the cell-attached configuration.

Results: Single channel activity from several members of the K2P family were recorded on each preparation, counting the number of patches with each subunit we carried out a quantification for each channel, so SCG neurons showed TRESK and TREK-2 channel activity while NG presented TRESK, TREK-1b, TREK-1a and TREK-2 conductances. Conductance, P₀ and dwell time were calculated for each subunit and the voltage dependency was determined.

Conclusions: SCG and NG cultured present several types of functional K2P channels in their membrane that contribute to maintain their resting membrane potential.

References:


This work was supported by grant CSD2008-00005 and BFU2008-02952 from the MICINN (Spain). XUNTA DE GALICIA (INBIOMED 2009/063 y IN845B-2010/148) and UNIVERSIDAD DE VIGO (00VI 131H 641.02). FEDER funds.
Connexins (Cx) that typically forms cell-to-cell channels mediating direct intercellular communication allow cells exchange ions and other molecules across the plasma membrane. Lastly, Cxs have been also enrolled in proliferation and differentiation control of progenitor cells. Here we explore the Cx expression and biological implications in neural precursor cells (epSPC) derived from adult rat spinal cord. epSPC significantly improve functional locomotor regeneration when transplanted after spinal cord injury (SCI). Moreover, FM19G11, a new chemical entity, intrathecally administrated in vivo, favors epSPC activation with improved functional locomotor recovery in a rat SCI model. FM19G11 and the injury-related process itself induce Cx 50 and 43 gene and protein expression in vivo and in vitro of epSPC cultures. epSPC results “activated” after SCI process (epSPCi) showing enhanced proliferative rates and better yields when oligodendrocyte-directed differentiation process is induced in comparison with epSPC from healthy donors. A relationship of Cx50 with self-renewal and differentiation potential of epSPC activated either by the injury (epSPCi) or FM19G11 treatment was explored. siRNA experiments for specifically knocking-down Cx50 confirm a direct regulation of stemness cell markers like Sox2 and Oct4 in undifferentiating cell growth conditions. Interestingly, both reprogramming process, self-renewal induction and directed-differentiation process involved Cx50 intervention. The expression of Cx43 and Cx50 during directed differentiation form epSPCi to oligodendrocytes exhibited an inverse tendency relationship to its expression levels with a fast and consistent sub-cellular location changes along the cell maduration process. Our observations demonstrate an involvement in vitro and in vivo of Cxs neuronal progenitor’s self-renewal and cell differentiation.

Supported by Spanish Consolider Ion Channel Initiative [CSD 2008-00005] MICINN grant
Spike timing-dependent plasticity (STDP) is a strong candidate synaptic mechanism involved in cortical development and map plasticity. In STDP, the temporal order and precise timing of pre- and postsynaptic action potentials (spikes) determine the direction and magnitude of synaptic change. Both timing-dependent long-term potentiation (t-LTP) and timing-dependent long-term depression (t-LTD) depend on NMDA receptors. How the same type of receptor can be involved in opposite changes in synaptic efficacy is not well understood. Whereas it is established that postsynaptic NMDA receptors are necessary for t-LTP, we investigated here whether presynaptic NMDA receptors are necessary for timing-dependent LTD and LTP in layer (L) 4-to-L2/3 excitatory synapses in mouse barrel cortex.

We used paired whole-cell recordings of synaptically-connected L4 and L2/3 cells. In five pairs, a pre-before-post pairing protocol was applied with 1 mM MK-801 in the presynaptic pipette. Robust t-LTP was induced (154±5%, n=5; p<0.01, t-test), of similar magnitude to that seen with extracellular stimulation (153±9%, n=5), suggesting that presynaptic NMDA receptors are not necessary for induction of t-LTP. In contrast, t-LTD was completely blocked when MK-801 was included in the presynaptic pipette (104±6%, n=6), whilst in pairs of cells without MK-801 this protocol induced robust t-LTD (76±6%, n=6; p<0.01, t-test), indicating that presynaptic NMDA receptors are necessary for t-LTD. The different sites of NMDA receptors necessary for induction of t-LTP and t-LTD may have important consequences for the computational operation of cortical microcircuits and map plasticity.

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Supported by MICINN grant BFU2009-10034
Role of Kv1.3 And Kv1.5 in the Phenotypic Switch of Human Vascular Smooth Muscle Cells.

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OBJECTIVE
Changes in the expression ratio of Kv1.3 and Kv1.5 channels associate with vascular smooth muscle cells (VSMCs) phenotypic modulation, due to differential effects of both channels on proliferation¹. In this study we explore whether the Kv1.5 to Kv1.3 switch is conserved in humans and the signaling pathways involved.

METHODS
Coronary, renal and uterine arteries and saphenous veins obtained through COLMAH biobank were used directly (contractile VSMCs) or explanted to get cultured VSMCs (proliferative phenotype). Kv1.3 and Kv1.5 mRNA expression levels were determined by real-time qPCR. Protein expression levels were analyzed by western blot, and functional characterization was carried out with patch-clamp experiments using K⁺ channels selective blockers. Proliferation of cultured VSMCs was determined with EdU incorporation assays.

RESULTS
qPCRs data determined that in all vascular beds Kv1.3:Kv1.5 ratio could be used to establish the phenotype of VSMCs. While Kv1.5 transcripts prevailed in contractile cells, Kv1.3 was predominant in proliferative cells. Western blot, together with patch-clamp experiments using specific blockers for Kv1.3 (PAP-1 and Margatoxin) and Kv1.5 (DPO), supported this observation. Moreover, PAP-1 and Margatoxin decreased PDGF-induced VSMCs proliferation. To explore the molecular mechanisms involved in the role of Kv1.3 during proliferation, cells were treated with blockers of different signaling cascades. ERK1/2 and PI3K-specific inhibitors decreased PDGF-mediated proliferation. PAP-1 inhibition was abolished with ERK1/2 inhibitors, but had an additive effect on the inhibition induced by PI3K and mTOR blockade.

CONCLUSIONS
Our results indicate that Kv1.3 promotes VSMC proliferation via ERK1/2 pathway. We propose that Kv1.3-selective inhibition represents a new therapeutical approach for the treatment of vascular occlusive diseases that could also reinforce current therapies based on mTOR blockade.

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Supported by grants R006/009 (ISICIII, Red Heracles), BFU2010-15898 (MICIN) and VA094A11-2 (JCyL). Human samples were provided by COLMAH-HERACLES (Coleccion de muestras arteriales humanas).
Presynaptic Silencing Mediated by Cannabinoid Type 1

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Cannabinoid receptors are the most abundant G protein-coupled receptors in the brain and they mediate retrograde short-term inhibition at both excitatory and inhibitory synapses, as well as long-term depression of synaptic transmission at many synapses. The induction of presynaptically silent synapses is a means of modulating synaptic strength, which is important for synaptic plasticity. Persistent activation of cannabinoid type 1 receptors (CB1Rs) mutes GABAergic terminals in hippocampal slices, although it is unclear if CB1Rs can also induce silencing at glutamatergic synapses.

We found that prolonged stimulation (10 min) of cannabinoid receptors with the agonist HU-210 induced silencing of previously active synapses in cerebellar granule cells, having transfected these cells with vGLUT1-pHluorin to visualise the exo-endocytotic cycle. Synaptic silencing was prevented by increasing cAMP levels with forskolin, suggesting that a decrease in this nucleotide initiates the silencing process in nerve terminals. Synaptic silencing was not prevented by activating the cAMP-dependent protein kinase (PKA), but it was blocked by activation of the Exchange Protein directly Activated by cAMP (EPAC). Electron microscopy revealed that silencing was associated with synaptic vesicle (SV) redistribution within the nerve terminal, which diminished the number of vesicles close to the active zone of the plasma membrane. Finally, by combining functional and immunocytochemical approaches, we observed a strong correlation between the release capacity of the nerve terminals and RIM1α protein content, but not that of Munc13-1 protein.

We conclude that overstimulation of cannabinoid receptors can silence glutamatergic nerve terminals.

Supported by MEC BFU2009-07092; MEC BFU2010-16974; ISCIII-MEC-RD06-0026; CAM-I2M2 S2011-BMD-2349
Identification of a PKC-modulated Cardiac Kv1.5+Kβ1.3 Channelosome

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Objectives: Kv1.5 channels are the primary channels contributing to the ultrarapid outward potassium current (I_{Kur}). The regulatory Kvβ1.3 subunit converts Kv1.5 current from a delayed rectifier with a modest degree of slow inactivation to channels with both fast and slow inactivation components. Previous studies have shown that inhibition of PKC with calphostin C abolishes the fast inactivation induced by Kvβ1.3.

Material and methods: In this study, we investigated the mechanisms underlying this phenomenon using electrophysiological, biochemical, and confocal microscopy approaches. To achieve this, we used HEK293 cells (which lack Kvβ subunits) transiently cotransfected with Kv1.5+Kvβ1.3 and also rat ventricular and atrial tissue to study native α-β subunit interactions.

Results: Immunocytochemistry assays demonstrated that these channel subunits colocalize in control conditions and after calphostin C treatment. Moreover, coimmunoprecipitation studies showed that Kv1.5 and Kvβ1.3 remain associated after PKC inhibition. After knocking down all PKC isoforms by siRNA or inhibiting PKC with calphostin C, Kvβ1.3-induced fast inactivation at +60mV was abolished. However, depolarization to +100 mV revealed Kvβ1.3-induced inactivation, indicating that PKC inhibition causes a dramatic positive shift of the inactivation curve. Finally, immunoprecipitation and immunocytochemistry experiments revealed an association between Kv1.5, Kvβ1.3, the receptor for activated C kinase (RACK1), PKCβI, PKCβII, and PKCθ in HEK293 cells and also in rat ventricular, but not atrial, tissue.

Conclusions: Our results demonstrate that calphostin C-mediated abolishment of fast inactivation is not due to the dissociation of Kv1.5 and Kvβ1.3. We have characterized a Kv1.5 channelosome in which Kv1.5, Kvβ1.3, RACK1, PKCβI, PKCβII and PKCθ physically and functionally interact. A very similar Kv1.5 channelosome was found in rat ventricular tissue but not in atrial tissue.

Supported by Ministerio de Ciencia e Innovación (MICINN) Grants SAF2010-14916, Ministerio de Educación y Ciencia-PR2003-0056, and Red Cooperativa de Enfermedades Cardiovasculares RECAVA FIS RD06/0014/0006 (to C. V.) and BFU2011-23268 and CSD2008-00005 (to A. F.).
Effects of CL888 on Kv4.3, Kv4.3/KChIP2b and Kv4.3/KChIP3 channels

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Kv4.3 generates the transient outward current (I_{to}) that plays an essential role in shaping the early phase of the cardiac action potential. These channels are regulated by a family of calcium-binding proteins called KChIPs. Four members of this family have been cloned. KChIP3 and KChIP2b bind to Kv4.3 in the endoplasmic reticulum and facilitate intracellular trafficking and membrane expression of the channel. In addition, both subunits regulate the gating of Kv4.3 channels, slowing inactivation and accelerating the recovery of the inactivation. CL888 is a molecule that induces alteration or disruption in the binding between KChIP1 and Kv4.3, modulating the conformation and function of the complex. The aim of our study was to determine the effects of CL888 on Kv4.3/KChIP3 and Kv4.3/KChIP2 complex, as well as on Kv4.3 alone.

KChIP3 and Kv4.3 channels were transiently expressed in CHO cells. Kv4.3/KChIP2b channels were stably expressed in CHO cells. Currents were recorded using the whole-cell configuration of the patch-clamp technique.

Block of Kv4.3/KChIP3 channels induced by CL888 was concentration dependent with an IC_{50} of 23 nM, whereas Kv4.3 and Kv4.3/KChIP2b was not. At 100 nM, degree of block ranged according to: Kv4.3/KChIP3>Kv4.3/KChIP2b>Kv4.3. In all 3 channel complexes, CL888 accelerated the inactivation kinetics of the current by decreasing the slow time constant (τ_s). Thus, for Kv4.3/KChIP3 the τ_s value changed from 20.6±1.6 ms to 17.0±1.5 ms (n=5, P<0.05); for Kv4.3, from 19.7±1.6 ms to 10.8±1.5 ms (n=4, P<0.05); and for Kv4.3/KChIP2, from 38.1±5.8 ms to 23.9±4.0 ms (n=5, P<0.05). CL888 did not affect the voltage dependency of steady-state inactivation of Kv4.3/KChIP3 channels. However, it accelerated their recovery from inactivation kinetics (τ_{re}=62.0±12.1 ms vs. 49.8±9.8 ms, n=6, P<0.05).

We conclude that CL888 binds to Kv4.3 channels and modulates the gating and the kinetics of these channels. In all channels analyzed, CL888 accelerates the inactivation kinetics. However, Kv4.3/KChIP3 complex results to be more sensitive than Kv4.3 and Kv4.3/KChIP2b. Therefore, the sensitivity of Kv4.3 channels to CL888-like drugs will vary depending of the associated regulatory subunits.

Supported by SAF2010-14916, Red Temática de Enfermedades Cardiovasculares (FIS RD12/0042/0019) and FIS (PI11/02459).
In rodent sensory neurons, acid-sensing ion channel 3 (ASIC3) has recently emerged as a particularly important sensor of non-adaptive pain associated with tissue acidosis as it occurs during an inflammatory process. However, little is known about the human ASIC3 channel, which includes three splice variants differing in their C-terminal domain (hASIC3a, hASIC3b and hASIC3c).

Human ASIC3a transcripts represent the main mRNAs expressed in both peripheral and central neuronal tissues (DRG, spinal cord and brain), where a small proportion of hASIC3c transcripts is also detected. Interestingly, we show that hASIC3 channels (a, b or c) are able to directly sense extracellular pH changes not only during acidification (up to pH5.0), but also during alkalization (up to pH8.0), an original and inducible property yet unknown. When the external pH decreases, hASIC3 display a transient acid mode with brief activation that is relevant to the classical ASIC currents, as previously described. On the other hand, an external pH increase activates a sustained alkaline mode leading to a constitutive activity at resting pH. Both modes are inhibited by the APETx2 toxin, an ASIC3-type channel inhibitor. The alkaline-sensitivity of hASIC3 is an intrinsic property of the channel, which is supported by the extracellular loop and involves two arginines (R68 and R83) only present in the human clone.

Human ASIC3 is thus able to sense the extracellular pH in both directions and therefore to dynamically adapt its activity between pH5.0 and pH8.0, a property likely to participate in the fine tuning of neuronal membrane potential and to neuron sensitization in various pH environments.

Supported by FIS PI08/0014, FIS PI11/01601, RD07/0062/0006 (Inst. Salud Carlos III, Spain); 2009SGR869 (Gen. Catalunya); Fondation pour la Recherche Medicale (FRM); the Association Francaise contre les Myopathies (AFM); the Agence Nationale de la Recherche (ANR) and the Fédération pour la recherche sur le cerveau (FRC).
Isoleucine 443 is a Molecular Determinant of Stereoselective Bupivacaine Block of Kv1.5 Channels

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Bupivacaine block of wild type (WT) Kv1.5 channels is stereoselective, being R(+)bupivacaine 9-fold more potent than the S(-) enantiomer. The molecular determinants of this stereoselective block were attributed to the interactions between bupivacaine and, at least, three residues located at the S6 segment (T507, L510 and V514). These amino acids at S6 helix face to the S5 one. Therefore, our hypothesis is that some S5 amino acids might be also involved in this stereoselective interaction. In this study we analyzed the effects of the enantiomers of bupivacaine on I443A mutant Kv1.5 channels (S5 segment).

I443A mutant Kv1.5 channels were transiently expressed in HEK293 cells and the currents generated were recorded using the whole-cell configuration of the patch-clamp technique.

R(+)-bupivacaine and S(-)-bupivacaine block of I443A Kv1.5 channels exhibited IC$_{50}$ values of 110±54 µM and 141±36 µM, respectively, indicating that I433A Kv1.5 block is not stereoselective. In these mutant channels, the degree of stereoselectivity ($\theta$) was abolished (9 versus 1.3). In fact, the apparent association rate constant dropped 2-fold in the presence of R(+)bupivacaine, while it increased 5-fold in the presence of the enantiomer S(-). These opposite effects on $k_b$ explain the loss of stereoselectivity observed on I443A Kv1.5 channels. Moreover, we observed that the interaction between both enantiomers and I443A Kv1.5 channels was not use-dependent as we observed in WT Kv1.5 channels.

We conclude that isoleucine at 443 position is a molecular determinant of stereoselective Kv1.5 bupivacaine block and the interaction between both enantiomers and I443A Kv1.5 channels was not consistent with an open-channel block mechanism.

Supported by SAF2010-14916, Red Temática de Enfermedades Cardiovasculares (FIS RD12/0042/0019) and FIS (PI11/02459).
Diethylamine Effects on Muscle-Type Nicotinic Receptors Resemble those of Charged Lidocaine

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Objectives: This work was aimed to determine the inhibitory effects of diethylamine (DEA), which resembles the hydrophilic moiety of the lidocaine molecule, on muscle-type nicotinic acetylcholine receptors (nAChRs), since lidocaine is partially protonated at physiological pH and causes multiple inhibitory actions on nAChRs (Alberola-Die et al., 2011).

Materials and methods: Purified Torpedo nAChRs were transplanted to Xenopus oocytes and currents elicited by ACh (IAChs), either alone or co-applied with lidocaine or DEA, were recorded at different membrane potentials. Besides, giant proteoliposomes bearing nAChRs were used to record single-channel IAChs (excised patch, inside-out configuration), in the presence or absence of DEA. Docking simulations were also carried out to screen the possible binding sites of these molecules to the nAChR.

Results: DEA reversibly blocked IACh in a dose-dependent way (IC50 close to 70 μM; Hill coefficient of 1), but without affecting the rate of IACh desensitization. IACh inhibition by DEA was mainly observed at negative potentials, suggesting an open-channel blockade. The electrical distance of DEA binding into the channel pore was similar to that reported for lidocaine (δ=0.3). Furthermore, DEA blocked nAChRs in the resting (closed) state, indicating additional binding sites for this molecule outside the pore. Thus, nAChR blockade by DEA resembled lidocaine effects when applied at low doses (below the IC50). Single-channel experiments confirmed that DEA blocked nAChRs by decreasing the open channel probability, though only when applied to the extracellular side.

Conclusions: Functional data and docking assays indicate that DEA and the hydrophilic region of lidocaine account for the open-channel blockade of nAChRs. Besides, lidocaine and DEA interacts with residues located outside the ion channel.

References:


This work was supported by grant CSD2008-00005 from the MICINN (Spain).
Lidocaine has Different Inhibitory Actions on Nicotinic Receptors Expressed by Mouse Superior Cervical Ganglion Neurones

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Objectives: Recently, we have described that the local anaesthetic lidocaine inhibits muscle-type nAChRs (composed by 2α, β, γ, δ subunits) by different mechanisms of action (Alberola-Die et al., 2011). The aim of this work was to characterize the effects of lidocaine on acetylcholine-elicited currents from mouse superior cervical ganglion (SCG) neurons, which mainly express pentameric α3β4 nAChRs.

Materials and methods: Mouse SCG cell culture was carried out as described previously (Martínez-Pinna et al., 2002). Neurons were voltage-clamped by using the perforated-patch method and IAChs were elicited by fast application of ACh (100-300 µM), either alone or in the presence of lidocaine (3-100 µM).

Results: IAChs were reversibly blocked by lidocaine in a concentration-dependent way (IC50 ≈ 45 µM; nH close to 1). The IAch blockade elicited by lidocaine showed certain voltage-dependency, being stronger at hyperpolarized potentials, which suggests an open-channel blockade. Lidocaine also blocked resting-state (closed) nAChRs, as evidenced by the increased inhibition caused by 12 s lidocaine application just before its co-application with the agonist. Furthermore, lidocaine enhanced IAChs desensitization when applied at concentrations close to the IC50 (or higher); however, this effect on IAch decay could not be observed at lower doses.

Conclusions: These results indicate that lidocaine inhibits nicotinic receptors expressed in autonomic ganglia neurones by different mechanisms (open-channel blockade, enhancement of desensitisation, and blockade of closed receptors), similarly as we have previously reported for muscle-type nAChRs microtransplanted to Xenopus oocytes.

References:


This work was supported by grant CSD2008-00005 from the MICINN (Spain).
Identification of a New Mineralocorticoid Receptor Agonist by Screening of a Pentameric Peptoid Library

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The mineralocorticoid receptor (MR) is a transcription factor of the nuclear receptor superfamily. Its main physiological ligand is aldosterone, a steroid hormone that controls electrolyte homeostasis, extracellular volume and blood pressure. The main targets of aldosterone are the epithelial sodium channel and other channels and transporters involved in transepithelial transport of sodium, potassium and protons. Pharmacological control of MR activity is important in handling resistant hypertension and heart failure. In this study, we screened a library of controlled mixtures of pentameric \(N\)-alkylglycines to search for modulators of MR. Receptor activity was assessed using a luminescent transactivation assay in COS-7 cells in the presence or absence of aldosterone. Out of twenty independent mixtures, only one gave a significant decrease in MR activity. However, six different mixtures potentiated the effect of aldosterone, although none increased MR activity per se. Deconvolution of the library led to screening of fourteen individual compounds. Compound #12 gave the highest activity, with a 75\% increase of aldosterone action in the presence of vehicle. The effect of this compound is time dependent and increases when cells are pre-treated for 24h before adding aldosterone. In summary, we have identified a new pentameric peptoid that behaves as an allosteric agonist towards MR.

Supported by Consolider SICI 2008-00005, BFU2010-16625 and SAF2011-30542-C01-01.
TRP ion channels family is represented by 85 members that can be organized by their sequence homology into seven subfamilies. Some members of these subfamilies play an important role in detecting temperature changes.

Within TRPV (vanilloid) subfamily, TRPV1 is the most studied member, and has been related with chronic pain, furthermore its pharmacological blockade and genetic deletion experiments have validated TRPV1 as a therapeutic target. Another member of the TRP family is TRPA1, which is activated by noxious cold and chemical compounds including allyl isothiocyanate (AITC), the pungent principle of wasabi and other mustard oils. TRPA1 appears to have a central role in the pain response but also it has been demonstrated that is essential for asthma [1]. TRP melastatin 8 (TRPM8) is activated by chemical cooling agents (such as menthol) or by temperatures between 28-15 ºC, mediating the detection of innocuous cold thermal stimuli. TRPM8 expression up-regulates has been suggested to play an important role in carcinogenesis and related with prostate cancer [2].

In this study was evaluated the biological activity of a new chemical library, through high throughput screening. We report here the identification of compounds presented a high blockade activity on TRPM8 and share common structure. These hits with notorious antagonistic effect were selected and observed in patch-clamp experiments performed in stable cell lines that expressed TRPV1, TRPM8 and TRPA1 to characterize more accurately their properties.

These new pharmacophoric scaffolds can be used as a hit to develop new compounds with better modulator properties interesting to the clinical field or as a research tool.

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Supported by Consolider-Ingenio 2010 (CSD2008-00005), MICINN (BFU2009-08346), MICCIN (BES-2010-037112), La Fundacion La Marato de TV3, Prometeo-GVA and SAF2009-09323.
Identification of Triazines as Novel Antagonists of Nicotinic Acetylcholine Receptors

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Acetylcholine is a neurotransmitter present in both the peripheral (PNS) and central (CNS) nervous systems. It is involved in a wide range of functions such as cortical activation, the enhancement of sensory perceptions when we wake up and in sustaining attention.¹ Nicotinic acetylcholine receptor (nAChR) subtypes having a wide diversity of composing subunits are widely distributed throughout both nervous systems, including several regions of the brain. Nicotine is the most potent agonist of some of the neuronal subtypes¹. These receptors play an important role in different neuropathological and physiological states, including Parkinson’s and Alzheimer’s diseases, pain, anxiety and tobacco dependency. nAChRs belong to the ligand-gated ion channels superfamily and they are composed of five homologous subunits symmetrically arranged around the ion pore¹.

In the present work, a group of 2,4,6-trisubstituted-1,3,5-triazines have been synthesized and tested as antagonists of α7 (present in PNS and CNS), α4β2 (predominant subtype in CNS) and α3β4 (predominant subtype in PNS) nAChR receptors. The synthesis of the 2,4,6-trisubstituted-1,3,5-triazines (Figure 1) was carried out employing the methodology developed in our research group² applying microwave-assisted chemistry techniques. The triazines were substituted at R₁ position with different amines containing electron donating and electron withdrawing groups in order to evaluate the effect of these moieties. The importance of the alkyl amine nature was studied at R₂ position as well. Inhibition tests of the response to acetylcholine were carried out either at 3 or 10 µM concentrations, as indicated for each compound in Figure 2 (bottom). Total inhibition activity was observed for all compounds with α7 nAChRs whereas the other subtypes were clearly less sensible. Inhibitory potency was not significantly different for three compounds that were studied in more detail. The most potent was AVR78 with an IC₅₀ value of 0.8 µM. Dose-response curves showed that inhibition was of the non-competitive type and voltage independent. This is in contrast what was previously observed with the TRPV1 receptor, where compound 8aA showed strong voltage dependency². In any case, we can conclude that trisubstituted triazines act as non-competitive antagonists of receptors with very different structures.

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Supported by Min. de Ciencia e Innovación (Spain) (grants BFU2008-02160, SAF2011-22802 and Consolider CSD2008-00005)
Preliminary Pharmacokinetic Data Obtained from the Treatment of Animals Models with Triazine 8aA, a Potent, Non Competitive TRPV1 Antagonist

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Ion channels are pore-forming proteins which are involved in the control of the voltage gradient across the cellular membrane by flow of ions when an electrochemical gradient is present. TRPV (Transient Receptor Potencial Vanilloid) is a family of transient receptor potential ion channels which are involved in signal transduction of thermal stimuli. In particular, the activity of TRPV1 (Figure 1), which is related to the transmission and modulation of pain as well as the integration of diverse painful stimuli, is potentiated by proinflammatory agents released upon tissue damage. Numerous TRPV1 antagonists have been identified as blockers of this receptor with high efficacy and potency but they exhibited non-desired side effects as hyperthermia. This general drawback has prompted the study of new, effective and non-toxic active antagonists.

In our research group, a small library of 2,4,6-trisubstituted-1,3,5-triazines has been synthesized and screened in vitro. These triazines act as uncompetitive TRPV1 antagonists at submicromolar concentration. Triazine 8aA (Figure 1), has been the most potent compound tested, exhibiting an IC₅₀ of 50 nM.

The potential of this compound in the in vitro tests has stimulated the development of in vivo studies in animal models. Mice populations have been treated with 8aA under different administration ways, intravenous and intraperitoneal, in a 1 mg/Kg dose and aliquots of plasma have been analyzed in different time intervals. In this communication, results on preliminary pharmacokinetic data obtained from these plasma samples will be presented. Funded by Consolider-Ingenio 2010 (CSD2008-0005); BFU2009-08346; PROMETEO/2010/046.

Characterisation of Induced Pluripotent Stem (iPS) Cell Derived Cardiomyocyte-like Cells

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Objectives: The Brugada Syndrome (BrS) is an inherited heart disease that is responsible for 20% of all sudden cardiac deaths. Fibroblasts from patients with BrS can be reprogrammed to generate induced pluripotent stem (iPS) cells which can then be differentiated to cardiomyocytes. The advantage of this model system is that it investigates the cell-line directly affected in the progression of the disease using cells that carry the patient’s exact genetic background.

Methods: Undifferentiated human embryonic stem (ES) cells and healthy control iPS cells were differentiated into cardiomyocyte-like cells using an unguided differentiation protocol (Xu et al, 2002). Beating clusters were characterised using immunocytochemistry, RT-PCR, edge detection and electrophysiology. Derived cardiac cells were then compared to primary cardiomyocytes isolated from human foetal and adult cardiac tissue.

Results: ES and iPS derived cardiomyocyte-like cells spontaneously contract and express a wide range of cardiac markers such as GATA-4, α-actinin and TroponinI. They express TTX resistant voltage dependent sodium channels and exhibit action potential triggered Ca^{2+}-induced-Ca^{2+}-release. Spontaneous contraction is a feature of immaturity as observed in cardiomyocytes isolated from human foetal cardiac tissue.

Conclusion: Although cardiomyocyte-like cells generated from iPS cells show some markers of immaturity, they can be used to not only effectively model Brugada Syndrome in patients with and without known genetic cause but also mutation carriers that show no symptoms. The iPS cell based model system also offers the most accurate and relevant setting for drug toxicity screenings.

References

Brugada Syndrome (BrS) is a life-threatening arrhythmogenic disease characterized with alterations of the sodium currents in the heart and associated with a high risk of Sudden Cardiac Death. Genetic alterations in SCN5A, which encodes the alpha subunit of the cardiac sodium channel (Na\textsubscript{v}1.5), are the most common cause of BrS, although they only explain 20-25% of the patients with BrS. We suggest that a dysregulation in SCN5A expression levels could be a new cause of BrS. The aim of this work is to study the mechanisms that regulate SCN5A gene at transcriptional level, and thus establish whether an alteration of these mechanisms could be associated with BrS.

Using online prediction programs we have identified binding sites for the transcription factor GATA-4 at the human SCN5A promoter. As an approach, we performed GATA-4 overexpression or knockdown experiments in H9c2 cells (a cardiac cell line derived from embryonic rat ventricle). The effect of GATA-4 on SCN5A promoter was analyzed in luciferase reporter experiments and real-time PCR. Our results show that the transcription factor GATA-4 acts as transcriptional activator of SCN5A promoter. We also observed that co-transfection of GATA-4 together with the p300 acetyltransferase further increases SCN5A expression. This suggests that GATA-4 transcriptional activity on SCN5A promoter could be regulated by p300-mediated acetylation. We have optimized Chromatin Immunoprecipitation (ChIP) to study GATA-4 binding to the SCN5A promoter in cardiomyocytes, as well as, the role of epigenetics modifications on the SCN5A promoter function.

In conclusion, our findings identify GATA-4 as a key regulator of SCN5A expression. This study will contribute to the understanding of a novel molecular biology-based mechanism of BrS.

Supported by:
Ministerio de Ciencia e Innovación. Study of the molecular mechanisms that regulate SCN5A expression and function (SAF2011-27627).

7PM-PEOPLE. Marie Curie International Reintegration Grant. Study of the molecular mechanisms that regulate SCN5A expression (PIRG07-GA-2010-268395).

Universitat de Girona. Estudi dels mecanismes moleculars que regulen l’expressió i la funció del gen SCN5A. Beques de Recerca UdG (BR2012/47).
The two-pore domain $K^+$ channel TRESK is expressed in dorsal root ganglion and trigeminal sensory neurons where, together with TREK-2, it carries about 80% of the total $K^+$ background current. Peripheral axotomy decreases TRESK expression, which likely contributes to enhance sensory neuron excitability after injury. Also, mutations in the channel have been linked to familial migraine with aura. Alkylamides like hydroxy-$\alpha$-sanshool and IBA (isobutylalkenylamide), which inhibit TRESK, produce tingling, cooling and pungent burning sensations in humans and nocifensive behaviors in rats. Pharmacological blockade or channel expression knockdown decreases the mechanical threshold to painful stimuli and produces nocifensive behaviors in rats. All these effects have implicated this channel in nociception and mechanotransduction.

To determine the role of TRESK in sensory transduction, we studied its mechanosensitivity in heterologous systems, F-11 cells and trigeminal neurons. Laminar shear stress increased TRESK currents by 22-30%. An increase in membrane tension induced by cell swelling (hypotonic medium) produced a reversible elevation of TRESK currents (39.9%). In contrast, cell shrinkage (hypertonic solution) produced the opposite effect. Membrane crenators or cup-formers produced equivalent effects. In trigeminal sensory neurons, TRESK channels were mechanically stimulated by negative pressure, which led to a 1.51-fold increase in channel open probability. TRESK-like currents in trigeminal neurons were additively inhibited by arachidonic acid, acidic pH and hypertonic stimulation, conditions usually found after tissue inflammation.

Our results show that TRESK possesses an intrinsic mechanosensitivity that depends on membrane tension. During tissue injury or inflammation, stimuli such as small osmotic changes will be able to modulate the channel, in addition to the inhibitory effect of inflammatory mediators like arachidonic acid or acidic pH. The combination of all these factors will down-regulate TRESK currents to enhance sensory neuron excitability.

Supported by grants from and Ministerio de Sanidad of Spain: FIS 08/0014; FIS PI11/01601 and by RETIC RD07/0062/0006 and 2009SGR869, Generalitat de Catalunya. JPG is supported by a Ramón y Cajal fellowship (RYC-2011-08589).
Excitotoxicity is one of the most important mechanisms contributing to neuronal damage and cell death in neurodegenerative diseases and ischemic events. Excess of the excitatory neurotransmitter glutamate activates NMDA receptors that play an important role in excitotoxicity because of its high permeability to Ca$^{2+}$. It has been suggested that the ensuing mitochondrial Ca$^{2+}$ overload may contribute to neuronal cell death in excitotoxicity. We have reported previously that a series of non-steroidal anti-inflammatory drugs (NSAIDs) may inhibit mitochondrial Ca$^{2+}$ overload. Here we studied directly whether NSAIDs may protect against NMDA acting on mitochondrial Ca$^{2+}$ handling.

Hippocampal cells from neonatal rat pups were cultured for several periods and used for monitoring of cytosolic and mitochondrial Ca$^{2+}$ by fluorescence and bioluminescence imaging, respectively. Cell death and apoptosis induced by NMDA were estimated by annexin and propidium iodide staining in the presence and the absence of different NSAIDs.

We found that NMDA induced a large increase in cytosolic and mitochondrial Ca$^{2+}$ in hippocampal neurons and these effects increased with culture time. Moreover, NMDA induced cell death and this effect also increased with culture time. A series of NSAIDs inhibited cell death induced by NMDA. This effect was achieved also by a structural analogue lacking anti-inflammatory activity. Finally, NSAIDs inhibited mitochondrial Ca$^{2+}$ uptake without affecting the rise in cytosolic Ca$^{2+}$ induced by NMDA.

We conclude that NSAIDs may protect against excitotoxicity in hippocampal neurons. This effect is probably independent of anti-inflammatory activity but related to effects on mitochondrial Ca$^{2+}$ handling.

*Supported by DIGICYT (BFU2009-08967) and by European Social Fund and Conserjería de Educación, Junta de Castilla y León (VA270A11-2).*
Ion Channels Expression in Mesenchymal Stem Cells of Human Wharton’s Jelly

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Background: The ion channels are protein transmembrane structures, which are essential for all cell functions including synaptic transmission, muscle contraction, hormone secretion, cell volume regulation, proliferation and cell differentiation. Each type of cells possesses each own “orchestra” of ion channels adequate for instant cell needs. It is growing understanding of their importance for cell physiology. However, there are few data on their expression and roles in Mesenchymal Stem Cells of Wharton Jelly Human Cord (hwMSCs). The purpose of the present study was to identify the main functional ionic channels present in these cells.

Methods: RT-PCR analysis of not synchronized hwMSCs was used to investigate the levels of mRNA expression. Ion currents were recorded by the patch-clamp technique.

Results: The calcium dependent K⁺ MaxiK and the anion pl-VDAC ion channels are the most abundant mRNAs in these cells. Other sodium, calcium and potassium channels are also expressed. Both inward and outward currents can be recorded in hwMSCs. The currents recorded are sensible to the external calcium and to the Cl⁻ channels inhibitor DIDS.

Conclusion: The data extend the knowledge about the types of ion channels and the level of expression in hwMSCs increasing a basic understanding of the biology of mesenchymal stem cells importance for future in vitro experiments and in vivo clinical investigations.
Pathogenic Mechanisms of two Novel connexin-47 Mutations Causing The Pelizaeus-Merzbacher-Like Disease

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Recessive mutations in the connexin-47 (Cx47) gene cause the Pelizeaus Merzbacher-like disease (PMLD), an early onset dys- and de-myelinating disorder that courses with nystagmus, impaired motor development, abnormal movements and progressive spasticity. Oligodendrocytes express Cx47 which can form "reflexive" channels intramyelin and intercellular channels between oligodentrocytes and between oligodendrocytes and astrocytes. This network of coupled cells is part of the "panglial syncytium" which has been implicated, among other functions, in the spatial buffering of K⁺ released during axonal activity. Previously we have analyzed the functional consequences of two novel PMLD mutations, the H252D and Y47H, and found that they impaired the formation of gap junction plaques and functional channels. Here we explore a second disease mechanism. We hypothesized that these PMDL mutants also would cause the disease by interfering with the functionality of precursors of complete channel (i.e., the hemichannels). Thus, our primary goal was to determine whether hemichannels of wild type Cx47 could be functional. Cx47 was expressed in unpaired Xenopus oocytes and we found that Cx47 was able to form functional hemichannels which were activated by depolarization of membrane potential, and opening is critically dependent on the external [Ca²⁺]. The open probability was very low at normal millimolar extracellular [Ca²⁺] but increased significantly by lowering [Ca²⁺] to micromolar range. At low [Ca²⁺], hemichannels activated upon depolarization, inactivated at higher positive potentials and were effectively closed by returning to negative potentials. Moreover, Cx47 hemichannel currents were blocked by acidosis (pH 6.0) and carbenoxolone (500 µM). Interestingly, oocytes expressing the H252D and Y47H mutants also exhibited hemichannel currents with similar, if not identical, properties of regulation by calcium and voltage. Thus, we conclude that these two mutations impair the formation of gap junction channels by a defect of docking and that they cause the disease by a loss of channel function but not by a hemichannel dysfunction.

Supported by grants of the Spanish Ministry of Science and Technology (SAF-2009/1164 and Consolider CSD2008-00005) and the Community of Madrid (Neurotec-P2010/BMD-2460)
Metabolic Status and Cardiac Potassium Channels Synthesis in Experimental Diabetes


Aims: The fast transient outward, $I_{to,fast}$, is the most extensively studied $K^+$ current in diabetic animals. Two hypotheses have been proposed to explain how type 1 diabetes reduces this current in cardiac muscle. The first one is a deficiency in channel expression due to a defect in the trophic effect of insulin. The second one proposes flawed glucose metabolism as the cause of the reduced cardiac potassium current. Moreover, little information exists about the effects and possible mechanisms involved in the effects of diabetes on the other repolarizing currents present in the human heart: $I_{to,slow}$, $I_K$, $I_{Ks}$, $I_{Kur}$, $I_{Kslow}$ and $I_{K1}$.

Methods: Cardiac action potentials and $K^+$ currents were recorded in ventricular cells isolated from control and streptozotocine- or alloxan-induced diabetic mice and rabbits. Channel protein expression was determined by immunofluorescence.

Results: Diabetes reduces the amplitude of $I_{to,fast}$, $I_{to,slow}$ and $I_{Kslow}$, in ventricular myocytes from mouse and rabbit, with no effect on $I_{ss}$, $I_K$ or $I_{K1}$. The absence of changes in the biophysical properties of the currents and the immunofluorescence experiments confirmed the reduction in channel protein synthesis. Incubation of diabetic myocytes with insulin or piruvate recovers current amplitudes and fluorescent staining. The activation of AMP-K reduces the same $K^+$ currents in healthy myocytes and prevents the piruvate-induced current recovery in diabetic myocytes.

Conclusion: Diabetes reduces $K^+$ current densities in ventricular myocytes due to a defect in channel protein synthesis induced by the activation of AMP-K secondary to a deterioration of the metabolic status of the cells.
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CPVT is an inherited disease characterized by the presence of ventricular tachycardia during emotional or physical stress that can cause sudden death. CPVT has been linked to genetic defects in two cardiac sarcoplasmic reticulum (SR) proteins, the type 2 cardiac ryanodine receptor (RyR2) and the cardiac calsequestrin (CASQ2). We have identified a large family from Canary Island with a long history of sudden death (32 patients with sudden death at young age 17±8 years). We analyzed biological samples from several of the deceased members and living relatives with a history of syncope. We identified a mutation in the position 1069 G>A of the RyR2 gene corresponding to the amino acid change G357S in the protein sequence. The overall goal of the present project was to analyze the functional properties of the mutant (MT) channels to identify the cellular and molecular pathogenic mechanisms associated to the G357S mutation. Our hypothesis is that the G357S mutation is responsible for CPVT in this family.

To test our hypothesis, we measured caffeine sensitivity of RyR2 wild type (WT) or MT in tetracycline-inducible HEK293 cell lines expressing either RyR2 or the G357S mutant. We also assessed their spontaneous overload induce calcium release (SOICR) activity in basal conditions and in the presence of forskolin which mimics catecholamine stress. Whilst caffeine sensitivity was not affected, the SOICR activity was slightly higher in cells expressing RyR2 MT protein. This effect was more prominent in the presence of forskolin, suggesting that G357S mutants are more sensitive to forskolin treatment.

Our findings suggest that the G357S mutation belongs to a new class of CPVT associated mutations. Experimental conditions that mimic beta-adrenergic stimulation unmask concealed pathogenic behavior of the RyR2 G357S mutant channel. These results provide a likely mechanism for the disease in this large family.

Supported by:
Instituto Carlos III, Ayudas Predoctorales de Formación en Investigación en Salud (PFIS). Mecanismos moleculares de la muerte súbita cardiaca asociados a la homeostasis del calcio intracelular (FI10/0045).
Centro Nacional de Investigaciones Cardiovasculares, CNIC-Translational. CNIC-03-2008.
Molecular determinants of BK channel function in neurovascular tissues of the heart

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Large conductance calcium-activated potassium (BK) channels are uniquely equipped to respond to both changes in membrane potential and in intracellular calcium concentration. One salient aspect of BK channels is the fact that the encoding gene Slo1 undergoes extensive alternative RNA splicing. These splice variants produce channels with diverse functional properties.

We are testing the hypothesis that BK channel alpha-subunit variants define the channel’s functional heterogeneity among neurovascular tissues within the heart. Specifically, we are interested in the characterization of porcine and canine intracardiac ganglia (ICG), and mouse aorta. Total RNA is purified from all the tissues and cDNA is synthesized using standard protocols. PCRs are performed to specifically amplify the regions for splice sites of interest are located, and the products are resolved in 2% agarose gels.

Our initial findings strongly suggest that both porcine and canine ICG express the splice variant known as STREX, since we clearly observed the presence of a band in the agarose gels with the molecular weight expected for the amplicon carrying STREX in both species. This insertion seems to be tissue specific since we did not observe the same pattern of bands when either porcine or canine brain RNA. In addition, the STREX amplicon was absent in mouse aorta.

We also hypothesize that BK channel variants from intracardiac neurons can be dynamically regulated, to provide a posttranscriptional control point for quantitative tuning of cell excitability. Indeed, the results obtained upon incubation of porcine ICG with increasing concentrations of cortisol suggest that STREX can be dynamically regulated by stress hormones in these ganglia.

Our observations constitute the first steps towards determining the molecular composition of BK channels in neurovascular tissues. A better definition of the composition and functional role of BK channel variants can be of potential clinical importance, not only because the channels may serve as specific therapeutic targets in cardiac disease, but also because functional or genetic alterations of particular variants may be contributing to currently unrecognized pathological conditions.
Hypoxic-induction of T-type Calcium Channels Involves HIF-1α and RhoA/ROCK

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Objectives: Voltage-gated T-type Ca\textsuperscript{2+} channels (T-channels) are expressed during embryonic development in ventricular myocytes but are undetectable in adult myocytes. Interestingly, T-channels are re-expressed in some pathological conditions associated with hypoxic episodes, although the underlying mechanisms are not known.

Materials and Methods: Cell cultures from adult and neonatal rat ventricular myocytes (NRVM) were used to study the effect of hypoxia on T-channels expression. mRNA levels were analyzed using RT-qPCR, and protein levels were studied by Western blot analysis. RhoA activity was measured using the G-LISA technique. T-channel currents were recorded using the whole-cell configuration of the patch-clamp technique. XTT Kit and BrdU immunocytochemistry were used to analyze cell proliferation.

Results: The Ca\textsubscript{v}3.2 subunit of the T-channels is highly induced by hypoxic conditions in dispersed cardiomyocytes (1% O\textsubscript{2}) as well as in a hypoxic animal model (8-9% O\textsubscript{2}, 24-48 h). The effect of hypoxia on Ca\textsubscript{v}3.2 gene expression is time- and dose-dependent, and is accompanied by an increase in the density of the T-channel currents recorded in patch-clamped NRVM. HIF-1α seems to be involved in the T-channel upregulation. Regulation by hypoxia is specific for T-type calcium channels, since it is not observed in other voltage-gated calcium channels. In addition, our results suggest that low oxygen conditions might modulate HIF-1α expression via the RhoA/ROCK signaling pathway. We show that hypoxia increased RhoA activity as well as RhoA and ROCK I protein levels. Furthermore, RhoA and ROCK inhibitors, reduced the hypoxia-induced HIF-1α expression and blocked the Ca\textsubscript{v}3.2 mRNA induction. Our results also suggest that the upregulation of T-channels by hypoxia, via HIF-RhoA/ROCK, may regulate proliferation in NRVM.

Conclusion: These results suggest that HIF-1α and RhoA/ROCK I are involved in the hypoxia-induced up-regulation of T-channels. This up-regulation may increase proliferation of NRVM, suggesting that this signaling pathway could play a role in the pathogenesis of ischemic cardiomyopathies.
Mutations affecting cardiac sodium current are often associated with arrhythmogenic diseases. While most of the mutations in Brugada Syndrome (BrS) are in the SCN5A gene, additional mutations in regulatory β subunits genes (SCN1B, SCN1Bb and SCN3B) have been found. Similarly, while 70-75% of congenital Long QT Syndrome (LQTS) cases are due to mutations in three major genes (SCN5A, KCNH2, and KCNQ1), a mutation related to this syndrome has been found in the SCN4B gene.

To better understand the role of β subunits in inherited arrhythmogenic diseases and to detect variants in the genes encoding for these subunits, we screened a population of both, BrS and LQTS patients, that did not have a mutation in any of the more common genes related to these diseases.

We sequenced the β subunit genes of 50 BrS and 40 LQTS cases. We found non-described missense mutation in SCN2B and in SCN1Bb in patients with BrS and LQTS, respectively. To investigate the effect of the mutant β2 on Naᵥ1.5, we performed studies in cells transiently transfected with Naᵥ1.5 together with WT or mutant β2 subunits. Electrophysiological analysis showed that mutant β2 subunit significantly reduced the peak IᵥNa density to 60.6%. We did not observe differences in the unitary current between Naᵥ1.5+β2WT and Naᵥ1.5+β2mut channels. Biotin experiments revealed lower membrane expression of Naᵥ1.5+β2mut. This suggests that the observed reduction in IᵥNa was due to an effect of the mutant β2 subunit on Naᵥ1.5 membrane expression.

Our study shows evidence for the association of a mutation in the sodium channel β2 subunit gene with BrS, pointing it out as a new candidate gene associated to this disease. In addition, ongoing research suggests that SCN1Bb is a putative new candidate gene for LQTS.

Supported by:
Instituto de Salud Carlos III, Ayuda Predoctoral de Formación en Investigación en Salud (PFIS). FI09/00336.
Centro Nacional de Investigaciones Cardiovasculares, CNIC-Translational. CNIC-03-2008.
Breathing is a primal neural process by which O$_2$ and CO$_2$ levels and pH are regulated. Brainstem neural circuits controlling breathing are organized as serially arrayed networks within neurons interact functionally by both chemical and electrical synapses. Electrical synapses are formed by cell-to-cell gap junction channels devoted to electrical signaling and metabolite exchange between neurons cells. Connexin-36 (Cx36) is the principal component of electrical synapses within distinct populations of brainstem respiratory nuclei, including the rhythmic neurons of pre-Bötzinger complex and CO$_2$/pH chemosensitive neurons of retrotrapezoid nucleus. Accordingly, we hypothesized that mice lacking of Cx36 (Cx36-KO) might present breathing related alterations. To obtain a complete characterization of the respiratory function in the Cx36-KO vs. wild type mice, breathing movements were continuously monitored during wakefulness and sleep, and the chemoreflexes in response to hypercapnia and hypoxia and the acid-base balance were studied.

We found that at resting wakefulness and during slow sleep epochs, wherein the respiratory rhythmicity is more robust, the ventilatory frequency of Cx36-KO mice was more irregular and somewhat higher. More dramatic differences were detected during epochs of REM sleep since there was a significant enhanced number of arrhythrias, apneas and arousals. Cx36-KO mice showed a reduced sensitivity to hypercapnia (2-8% CO$_2$) while the ventilatory response to hypoxia (10 % O$_2$) is exacerbated. The study of acid-base balance from blood and urine samples revealed a moderate and chronic respiratory acidosis. In vivo recording of pH in cerebral parenchyma also showed a greater acidification in the Cx36-KO mice. The results clearly indicate that mice lacking of Cx36 have an abnormal respiratory phenotype and suggest that electrical synapses can be implicated in the central chemoreception and in the generation of rhythmic breathing patterns.

Supported by grants of the Spanish Ministry of Science and Technology (SAF-2009/1164 and Consolider CSD2008-00005) and the Community of Madrid (Neurotec-P2010/BMD-2460)
Differences in Cell Proliferation and Store-Operated Ca\textsuperscript{2+} entry between Normal Colonocytes and Colorectal Adenoma Cells

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Intracellular Ca\textsuperscript{2+} plays a critical role in control of short-term and long-term cell functions including cell proliferation. In particular, the store-operated Ca\textsuperscript{2+} entry pathway (SOCE), that is activated by Ca\textsuperscript{2+} release from intracellular stores and modulated by mitochondria, has been involved in cell proliferation. In some cell types, the molecular basis of SOCE involves Stim1, an endoplasmic reticulum Ca\textsuperscript{2+} sensor and Orai1, a component of the Ca\textsuperscript{2+}-release activated current (CRAC). Recent data suggest that increased proliferation and survival of tumor cells may rely on remodeling of Ca\textsuperscript{2+} channels. In addition, pharmacological inhibition of SOCE may prevent tumor cell proliferation. Here we aimed at investigating remodeling of SOCE and its molecular players Stim1 and Orai1 in human colon adenocarcinoma cells relative to normal colonic mucosa cells.

Hemocytometry and calcium imaging was used for testing cell proliferation and SOCE in human colon adenocarcinoma cells (HT29 cells) and human normal colonic mucosa cells (NCM460 cells). Quantitative RT-PCR and western blotting were employed in the same cells for assessing expression of SOCE molecular players Stim1 and Orai1.

We found that colon carcinoma proliferated at a higher rate than normal colon mucosa cells. This functional difference correlated with increased SOCE and mitochondrial Ca\textsuperscript{2+} uptake in tumor cells relative to normal, non transformed cells. Interestingly, tumor cells expressed more Orai1 and Stim1 mRNAs and protein than normal cells suggesting that increased SOCE and cell proliferation characteristic of tumor cells are related to increased expression of CRAC molecular components.

Supported by DIGICYT (BFU2009-08967) and Junta de Castilla y León, Spain.
Expression, Function and Composition of L-Type Calcium Channels in a model of Essential Hypertension

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OBJECTIVE
In vascular smooth muscle cells (VSMCs), contraction is controlled by the intracellular calcium concentration \([\text{Ca}^{2+}]_i\). Four important components keep internal calcium at tight levels: L-type calcium channels (LTCCs), Ryanodine Receptor channels (RyR), resting membrane potential (Em) and large-conductance \(\text{Ca}^{2+}\)-activated \(\text{K}^+\) channels (BKs). Essential hypertension associated with an increasing \([\text{Ca}^{2+}]_i\), that has been linked to an increase of \(\alpha_{1C}\) subunit of the \(\text{CaV}_{1.2}\). Here, we studied the functional expression of LTCCs in mesenteric arteries VSMCs from normotensive (BPN) and hypertensive (BPH) mice.

METHODS AND RESULTS
RT-PCR analysis revealed a decrease in the \(\alpha_{1C}\) and \(\beta_2\) mRNA expression in BPH and a significant increase in the \(\alpha_2\delta\) subunit. Moreover, functional characterization of LTCCs currents with patch-clamp techniques showed that LTCCs current density was significantly decreased in BPH cells. Organization of the LTCCs was studied by direct observation of calcium influx “sparklets” with total internal reflection fluorescence (TIRF) microscopy. VSMCs LTCCs clusters operating in high open probability were significant larger in BPH mice. “Sparklets” amplitude histograms suggested that composition of the LTCCs subunits may be different between our two strains. To test this hypothesis, the composition of the LTCCs subunits was evaluated in a heterologous system using gabapentin, an antagonist of the \(\alpha_2\delta\) subunit. Gabapentin dose-response relationship indicated similarities between \(\alpha_{1C}+\alpha_2\delta+\beta_2\) transfected HEK and BPH and between \(\alpha_{1C}+\alpha_2\delta\) and BPN.

CONCLUSIONS
We found a decreased mRNA expression and a lower LTCCs current density in BPH. However, LTCCs cluster from BPH were composed of a larger number of channels and showed higher activity at rest. We postulated that the different subunit composition of LTCCs in BPN and BPH could account for these differences.

Supported by grants from ISCIII (R006/009, Red Heracles), MICIN (BFU2010-15898) and JCyL (VA094A11-2).
Role and Expression of Kv1.5 in Human B Lymphocytes


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Voltage-dependent K⁺ channels (Kv) control action potentials and membrane resting potential in nerve and muscle. In addition, Kv play a pivotal role in the leukocyte physiology. Numerous Kv blockers have demonstrated that Kv participate in the cell cycle progression. Leukocytes have a limited repertoire of Kv. Although Kv1.3 is major in T-lymphocytes, macrophages also express Kv1.5, which controls many physiological processes. Concerning B-lymphocytes, scarce information is available. The aim of the present work was to analyze the Kv isoforms expressed in B-cells and to characterize any putative physiological role. By pharmacological treatment and electrophysiological recordings we report that, unlike Jurkat T cells, human Ramos and Raji B-lymphocytes express of both, Kv1.3 and Kv1.5 similarly to macrophages. Blockage of Kv channels by specific antagonists inhibited cell proliferation, which is of physiological relevance in lymphocytes. In addition, by lentiviral transduction of shKv1.5, which produced a knock-down of Kv1.5, we elucidate further the role of the channel in the B cell physiology by analyzing proliferation, migration and cell volume. Because Kv1.3 and Kv1.5 channels modulate proliferation and activation of different mammalian cells, these proteins have been analyzed by RT-PCR, immunoblotting and immunocytochemistry in a number of cell lines, lymphoid tissues, tumors and cancer cells from mouse and human samples. In most cancers, the expression patterns of Kv1.3 and Kv1.5 are remodeled. In addition, a correlation has been established between Kv1.5 abundance and grade of tumor malignancy (abundance of p53 and Ki67). Because potassium channels may play a pivotal role in tumor cell proliferation, these proteins should be taken into account when designing new cancer treatment strategies.

Supported by BFU2011-23268 and CSD2008-00005 (MINECO, Spain).
Epithelial Sodium Channel (Enac) Plasma Membrane Turnover is Modified in Channels Containing δ Subunits

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Epithelial Na\(^{+}\) channel (ENaC) formed by alpha, beta and gamma subunits plays a key role in Na\(^{+}\) reabsorption across tight epithelia. Strict control of ENaC activity is essential for extracellular volume homeostasis. ENaC endocytosis is controlled by Nedd4-2 binding to PPxY motifs in the C-termini of beta and gamma subunits and subsequent ubiquitination of channel subunits. A fourth ENaC subunit, named delta, can interact with beta and gamma to form channels that are also regulated by ubiquitination. In this study we compared the trafficking of alpha/beta/gamma and delta/beta/gamma channels in Xenopus oocytes.

ENaC currents from different subunit combinations were quantified by two electrode voltage clamp (TEVC) in Xenopus oocytes. Fluorescence recovery after photobleaching (FRAP) was measured in a confocal (Olympus Fluoview1000) to quantify membrane insertion rates in the oocyte membrane under different conditions.

The use of brefeldin A to block the insertion of newly synthesized channels to the membrane showed a higher endocytosis rate for alpha/beta/gamma than for delta/beta/gamma. FRAP experiments were then used to study channel membrane insertion. The results indicate that the insertion rate of alpha/beta/gamma is higher than that of delta/beta/gamma.

Our data suggests that delta modifies ENaC turnover in the membrane and thus plays a dominant role in the regulation of channel trafficking.

Supported by Spanish Ministry of Science (grants Consolider CSD2008-00005, BFU2010-16265 and FIS-PS09/00406).