monitoring the motion of amino acids in real time, we propose the possibility of the thermal effects on the channel molecular dynamics, what that is remained to be further studied.

3457-Pos Board B504

Novel constitutively active non-store-operated Ca2+ current in T lymphocytes

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The Ca^{2+}-release-activated Ca^{2+} (CRAC) channels is the only known mechanism mediating Ca^{2+} entry in T cells. However, using Mn^{2+} quench of Fura-2 fluorescence we observed a constitutive divalent cation influx in the absence of stimulated store-operated Ca^{2+} entry in Jurkat T lymphocytes. Suppression of CRAC channels activity either with blocking concentration of La^{3+} or by expression of dominant-negative Orai1 mutant did not affect the rate of constitutive Mn^{2+} quench. These data suggest the existence of an additional non-store-operated mechanism mediating Ca^{2+} entry in T lymphocytes. Consistently, a constitutively active current was recorded in metabolically intact T cells using perforated-patch technique. Whole cell and perforated patch experiments revealed that in the presence of extracellular Ca^{2+} both constitutively active and CRAC currents displayed inwardly rectifying current-voltage relationship, positive (> 50 mV) reversal potential, and were enhanced by increased concentrations of extracellular Ca^{2+}. However, when the divalent cations were removed from the extracellular solution, the monovalent CRAC current displayed fast time-dependent inactivation, whereas the monovalent constitutively active current exhibited time-dependent activation and lack of inactivation. Equimolar substitution of Na^+ with Cs^+ in Ca^{2+}-free solution reduced the amplitudes of monovalent CRAC current and constitutively-active current by > 90 % and < 40 % respectively. Taken together, these data indicate that the CRAC and constitutively active currents are carried via different types of Ca^{2+}-selective channels. We speculate that in T lymphocytes the constitutively active Ca^{2+} entry channels may supply Ca^{2+} for maintaining resting cytosolic Ca^{2+} levels and/or for store refilling at unstimulated conditions. Supported by AHA Grant-in-Aid 0755086Y to A.F.F

3458-Pos Board B505

The Role of Ion Channels in Differentiating Chondrocytes

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Cartilage is an important load-bearing component of the skeleton of vertebrates. This tissue has similar composition to other members of the connective tissue family: major constituents are cells, the chondrocytes, and the surrounding extracellular matrix; however, it is also unique being avascular. Chondrocytes are non-excitable cells and little is known about their plasma membrane ion channels. The aim of our study was to identify ion channels and establish their roles in differentiating chondrocytes.

Our *in vitro* chondrogenesis model system is a high density mesenchymal cell culture, in which chondroprogenitor cells are isolated from limb buds of chicken embryos. Using whole-cell patch-clamp we have detected voltage-dependent ionic currents in these differentiating cells, whose amplitude depended on the time elapsed since isolation. An outward current was present in chondrocytes within 1-2 days of isolation, while an inward current gradually replaced it about 2 days after isolation. Using ion substitution experiments we identified the channels responsible for the currents as voltage-gated K^+ and Na^+ channels, respectively.

The average amplitude of the Na^+ current in cells during days 3-4 following isolation was -294 +/− 22 pA at 0 mV. The current inactivated with a time constant of tau = 0.59 +/− 0.04 ms. The voltage-dependence of steady state activation and inactivation were also determined yielding $V_{1/2}$ values of -38 and -72 mV. Tetrodotoxin reversibly blocked the current with a $K_d = 12$ nM. The results of planned molecular biological experiments combined with our biophysical and pharmacological data will be used to identify the channel. The characterization of the K^+ channel is presently underway.

In many cell types changes in ion channel expression are associated with differentiation, thus our long-term aim is the clarification of the role of these channels in chondrogenesis and its potential clinical consequences.

3459-Pos Board B506

A Gadolinium-Sensitive Non-Specific Cation Channel In Canine Articular Chondrocytes

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Non-specific cation channels are present in a number of cell membranes and can be activated by diverse cellular stimuli, allowing mono- and divalent cations to cross the cell membrane (Sanchez & Wilkins 2003).

In the present study we used both inside-out and whole-cell patch clamp electrophysiology to characterise the predominant ion channel in potassium free solutions.

Isolated chondrocytes were cultured for 7 to 9 days in Dulbeccos Modified Eagles Medium with 10% Foetal Calf Serum. Recording was carried out on first to third passage cells. For single channel data, membrane potential (*Vm*) was calculated as *Vm = -Hp-Vj* where *Hp* was the holding potential and *Vj* the calculated junction potential. Data are expressed as mean ± standard error.

Single-channel activity reversed at a membrane potential of 3 ± 2mV (n = 5) in the presence of 196mM internal and 155mM external Na^+, indicative of a non-specific cation channel. Mean slope conductance of the channel was calculated to be 67 ± 5pS (n = 5). This channel activity was seen in 53% of patches (32/61), with mean open probability of 0.6 at −40mV. 100μM gadolinium III reduced this open probability by 75 ± 9%.

In identical solutions the predominant whole-cell current showed a reversal potential of 1 ± 5mV. 100μM gadolinium III inhibited whole-cell current by 85 ± 7%. The whole-cell current exhibited weak voltage sensitivity with Boltzmann parameters for slope and half maximal activation of $k = 83$mV and $V_{1/2} = -38$mV.

The ion channels identified in these electrophysiological experiments may underlie the gadolinium-sensitive stretch-activated increases in calcium observed by Guilak *et al* (1999) in bovine tissue.

3460-Pos Board B507

Stim1 and Orai1 Mediate CRAC Currents and Store-Operated Calcium Entry Necessary for Endothelial Cell Proliferation

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Recent breakthroughs in the store-operated calcium (SOC) entry pathway have identified Stim1 as the endoplasmic reticulum (ER) calcium sensor and Orai1 as the pore forming subunit of the highly calcium selective CRAC channel. Previous studies have suggested that endothelial cell (EC) SOC is encoded by members of the Canonical Transient Receptor Potential (TRPC) channel family, either TRPC1 or TRPC4. Here we show that passive store depletion or receptor activation by thrombin or VEGF activates SOC entry pathway in primary EC with classical SOC pharmacological features. EC possess the archetypical store-depletion activated CRAC current. By amplifying currents in divalent free bath solutions, we show that EC CRAC has similar characteristics to that recorded from RBL cells, namely a similar time course of activation, sensitivity to 2-APB and low concentrations of lanthanides, the same inwardly rectifying I/V relationship, very positive reversal potential, and large sodium currents displaying the typical phenomenon of depotentiation. RNA silencing of either Stim1 or Orai1 essentially abolished SOC entry and CRAC currents in EC which were rescued by ectopic expression of either Stim1 or Orai1, respectively. Surprisingly, complete knockdown of either TRPC1 or TRPC4 proteins had no effect on SOC entry in EC. Smaller CRAC current densities in EC compared to those recorded in RBL cells were due to lower expression of Stim1. Ectopic expression of Stim1 in EC increased their CRAC currents to a size comparable to those in RBL cells. Knockdown of either Stim1, Stim2 or Orai1 inhibited EC proliferation and caused cell cycle arrest at S and G2/M phase, although Orai1 knockdown was more efficient than that of Stim1. These results are first to establish the requirement of Stim1/Orai1 in the endothelial SOC pathway necessary for proliferation.

3461-Pos Board B508

Androgens Stabilize HERG Potassium Channel Protein Via Stimulation Of Androgen Receptor Variant Ar45

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Proarrhythmic drugs induce long QT syndrome more frequently in women than men. The present study was designed to determine whether androgens regulate the function and expression of the human ether-á-go-go-related gene (HERG) encoded K^+ channel, which is largely responsible for determining the QT interval. In a concentration-dependent manner (10^{-9} to 10^{-6} M for 24 h), 5α-dihydrotestosterone (5α-DHT) increased HERG protein abundance in HEK293 cells stably expressing HERG in the presence of co-expressed cardiac androgen receptor variant (AR45). The elevation of HERG protein was seen in ER, Golgi and plasma membrane without clear preferential colocalization. Co-expression of the more common form of the androgen receptor did not confer 5α-DHT augmentation of HERG protein. Proteasome inhibitors, N-acetyl-L-leucyl-L-

leucyl-L-norleucinal and MG132 prevented the 5α-DHT-dependent enhancement of HERG as did the lysosome inhibitor, bafilomycin A1. Consistently, the cycloheximide-based protein chase study showed that 5α-DHT prolonged HERG protein half-life. 5α-DHT/AR45 signaling induced phosphorylation of extracellular signaling regulated kinase (ERK1/2). Blockade of ERK1/2 with PD98059 and U0126 prevented the effect of androgen on HERG protein abundance. Functional studies showed that 5-DHT treatment for 24 h increased HERG K^+ current density in CHO cells co-transfected with cDNAs of AR45 and HERG channels. Moreover, 5α-DHT also increased ERG protein abundance in isolated rabbit cardiac myocytes. In conclusion, these data provide evidence that stimulation of AR45 receptors by androgens upregulates HERG K^+ channel abundance and activity mainly through stabilizing HERG protein in an ERK1/2 dependent mechanism and suggest a mechanism to explain the sex difference in the long QT syndrome.

3462-Pos Board B509

Insights into the Ion Selectivity Mechanism of CNG Channels from Mutants of NaK: Structural and Functional Studies

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Cyclic nucleotide-gated (CNG) channels are non-selective cation channels that play crucial roles in visual and olfactory signal transduction. They are members of the tetrameric cation channel family that include voltage-gated K^+, Na^+ and Ca^{2+} channels. However, while other members exhibit high degree of ion selectivity, CNG channels are noted for their lack of specificity. CNG channels conduct all alkali metal ions and some alkaline earths, most notably Ca^{2+}. How the CNG channel pore can conduct these various cations which have substantially different ionic radii and formal charges is not well understood. Here we report high-resolution crystal structures of mutants of the NaK channel that mimic the selectivity filter of CNG channels, along with supporting functional analyses. Within the NaK selectivity filter (^{63}TVGDGNFS^{70}) the DGNFS sequence was replaced with ETPP, ETPT and DTPS, each of which represent a CNG α-subunit sequence. The mutant structures exhibit selectivity filter architecture and ion binding profiles different from either NaK or K^+ channel structures, having three ion binding sites in their selectivity filters. Two of the sites correspond to sites 3 and 4 in KcsA and NaK, while the third site corresponds to site 2 in KcsA, but is a vestibule in NaK. Similar to CNG channels these mutants exhibit calcium binding, which depends on the presence of the conserved acidic residues (E or D). Mutating the acidic residues on these mutants to neutral residues (E◇Q or D◇N) abolishes calcium binding. Functional analyses using Rb-86 flux assay revealed ion conduction behavior similar to CNG channels. These results provide strong evidence that these NaK mutants exhibit the same properties of CNG channels in ion conduction and selectivity and their structures provide insight into understanding ion selectivity in CNG channels.

3463-Pos Board B510

Water Dominated Ions Stability and Conduction in NaK Channel

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Water plays an important role in ion channels. It stabilizes ions in the central cavity and accompanies them to permeate through the channel, and it also participates in processes of ion selection. Here we find four water grottos connecting with the vestibule of the NaK selectivity filter, and they form a vestibule-grotto (V-G) complex in a plane perpendicular to the ion conducting pore. Molecular dynamics (MD) simulations show that water can penetrate and escape the grottos from the extracellular water pits above the grottos around the extracellular entrance, and two aromatic residues Tyr55 and Phe56 serve as a gate between the grottos and water pits. In the rest state, water molecules are confined in the vestibule and grottos and seldom exchange between them, and they have little impact on the K^+ ion binding states in the selectivity filter. While in the active state, the water molecules in the V-G complex become highly activated and they can flow easily between the vestibule and grottos. MD and free energy calculations show that the water molecules moving in the V-G complex hydrate and stabilize ions in the filter and serve as a valve in conveying ions through the vestibule for controllable ion permeating. The existence of the grottos and the simple and beautiful structure-function correlation of the hydration valve can be expected in the whole family of CNG channels, which function in our photoreceptors and olfactory cells.

3464-Pos Board B511

Functional investigation of the light-gated Channelrhodopsin

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The retinal proteins Channelrhodopsin-1 and −2 (ChR-1 and −2) from *Chlamydomonas reinhardtii*, which were first described as light-gated ion channels by Nagel *et al.* in 2002 and 2003, emerged in the last few years as advantageous tools. Since they open up rapidly after absorption of a photon and permeate ions like sodium or calcium, Channelrhodopsins are already used for non-invasive excitation of excitable cells in culture as well as in living tissue.
Together with other retinal proteins they share a 7-transmembrane helix motif where the retinal chromophore is covalently linked to the protein via a protonated Schiff base. Recent investigations by Bamann *et al.* (2008) predicted a photocycle with at least 4 photointermediates, all coupled to the channel function. But little is known about the mechanism that infers the properties of the ion channel or the channel pore, especially the different permeability coefficients between a series of cations and the strong inward-rectifying behaviour of the photocurrents is not fully understood.
Here we present a detailed functional characterisation by patch-clamp measurements on HEK293 cells stably expressing Channelrhodopsins. We could show that the inward rectifying properties are associated with the availability of cations and therefore predict cation binding to the protein. These results are discussed in relation to the hypothetical structure of the Channelrhodopsin and a putative cation binding site.

3465-Pos Board B512

Mutations in Cys 128 cause extreme decelerations of the Channelrhodopsin-2 kinetics

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Channelrhodopsin-2 (ChR2) triggers the phototaxis of the green algae *Chlamydomonas reinhardtii*. Amino acids 1-315 form a Bacteriorhodopsin (BR)-like heptahelical membrane domaine, which comprises the ion permeability and the covalently bound retinal chromophore. Depending on the electrochemical gradients, protons and cations are conducted in both directions upon light activation of ChR2. However, only little is known about the residues that determine the channel function, while proton pumping BR has characterized in almost any detail. In order to ensure a unidirectional charge transfer, BR goes through a photocycle with separated proton uptake and release in which only one proton is pumped. In contrast, Channelrhodopsins conduct hundreds of ions during each cycle. Hence, we deduce that these proteins are approaching a defined photointermediate which forms an open channel pore, i.e. the conducting state. By Two Electrode Voltage Clamp (TEVC) measurments in *Xenopus* oocytes, we identified an amino acid that plays a crucial role in this process. Mutation of C128 in Helix3 to Thr, Ala or Ser, decelerates the ChR2 kinetics dramatically. For instance, the on-kinetics of C128A is 10 times slower and the off-kinetics even 2000 times slower compared to the wilde type. In addition, we show that cells expressing these mutants are more than 300 times more light-sensitive than ChR2-WT and that they can be used as photo-switches. In BR, a corresponding threonine (BR-Thr90) is located near the 11-12 position of the retinal and is involved in the rearrangement of the α-helices during the photocycle[1]. We conclude that Cys 128 is critical for both fast opening anf fast closing of the of the ion channelpore.

1. Joh, N. H., Min, A., Faham, S., Whitelegge, J. P., Yang, D., Woods, V. L., and Bowie, J. U. (2008) *Nature* **453**, 1266-1270

3466-Pos Board B513

Four Intrinsic Aqueduct Orifices Outstretched from the Central Cavity Facilitate Potassium Channels Gating

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Potassium channels enable K+ ions to flow selectively across cell membrane through a central pore. The mechanisms of ion selectivity and channel gating have long been the attractive secrets. The breakthrough in determination of the structure of the KcsA potassium channel (Science 280, 69-77, 1998) has raised a high tide in structure and function study, but channel gating still remains a long secret. The core structure of K+ channels was found to be highly conserved, and constructed of an inverted teepee with a large water-filled cavity at center and the well studied selectivity filter at its wide end. Here we find four aqueduct orifices outstretched from the cavity and perpendicular to the central pore, leading to shape of a swastika or Greek Fleurée Cross, having subtle gating function. We demonstrated by systematical molecular dynamics simulations that water molecules flowing in the orifices can harmonize the space changing in the cavity to reduce the opening resistance significantly, and blocking the aqueduct orifices makes the intracellular entryway difficult to be opened. This is strongly supported by existed mutation experiments. Homology analyses of all available pore structures and amino acid sequences of K+ channels show that the aqueduct orifices are intrinsic structure feature to the whole potassium channel genre, but their size and conformation are less conserved among different subfamilies, shedding light on their functional diversity.