GABA inhibitory post-synaptic currents (mIPSCs) from cultured cerebellar granule cells under varying pH and proton buffering conditions. We found an inverse relationship between extracellular pH and mIPSC amplitude and charge transfer, resulting in over a 100% increase in size of events recorded at pH6.8 vs. pH8.0. Acidification also slowed the kinetics of rise time and fast component of decay, while speeding the slow decay component. We find that lowering the pH buffering capacity of the extracellular solution from 24 to 3mM HEPES at pH7.4, results in a similar enhancement of mIPSC size, mimicking changes in kinetics induced by acidification. The effects of diminished buffering capacity on mIPSC were negated by lowering extracellular pH to 6.8. To probe these effects with physiological buffers, we measured mIPSCs using 24mM of bicarbonate and compared them with those recorded in 24mM bicarbonate supplemented with 10mM HEPES . We found that physiological concentrations of bicarbonate produced mIPSCs that were similar in size and kinetics to those found with 3mM HEPES and were similarly altered with addition of HEPES, confirming the physiological relevance of our findings. To determine the possible contribution of Na+/H+ exchanger to synaptic acidification we inhibited the exchanger with amiloride (20μM), and in a parallel set of experiments replaced extracellular sodium with lithium. Both of these treatments caused changes in mIPSCs that mirrored increased buffering capacity, and the effects were negated by acidification to pH6.8 or by increasing HEPES buffering capacity to 24mM. We conclude that GABAergic synaptic pH in vivo may be quite labile and subject to rapid and pronounced acidification from the Na+/H+ exchanger with the net effect of enhancing synaptic transmission.

2434-Pos Board B404

Voltage-dependent Gating Of Wt And D177a Eaat4-associated Anion Channels

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Excitatory amino acid transporters (EAATs) are not only secondary-active glutamate transporters, but function also anion-selective channels. Ryan and Vandenberg (JBC, 279: 20742-20751, 2004) recently demonstrated that mutations in the interlinker between transmembrane domain 2 and 3 of EAAT1 affect selectivity of EAAT anion channels suggesting that this domain forms part of the anion-selective pore. We here study the effect of a point mutation within this region, D117A, on anion channels associated with another EAAT isoform, EAAT4. WT and D117A EAAT4 were expressed in tsA201 cells and studied through whole-cell patch-clamping under a variety of conditions. WT EAAT4 anion channels conduct anions over the whole voltage range and exhibit two types of voltage-dependent gating, one activated by membrane hyperpolarisation, and another one activated during membrane depolarisation. Glutamate shifts depolarisation- and hyperpolarisation-induced gating to more negative potentials in a dose-dependent fashion. At saturating glutamate concentrations, both gates are active in a physiological voltage range. Only in the presence, but not in the absence of glutamate, gating of WT anion channels also depends on anion concentrations on both membrane sites. External anions shift the activation curve of both gating processes to more negative potentials, whereas increasing concentration of internal anions have the opposite effects. D117A has dramatic effects on permeation, gating and glutamate dependence of EAAT4 anion channels. The amplitude of D117A EAAT4 anion currents is not affected by glutamate. At symmetric anion concentrations, D117A EAAT4 anion channels are strictly outwardly rectifying, in clear contrast to WT EAAT4 that effectively conduct anions in both directions. Moreover, D117A EAAT4 channels exhibit only a single gating process, activated by membrane depolarization. Gating of D117A EAAT4 is not affected by glutamate. Our results suggest a crucial role of D117 for the function of EAAT anion channels.

Ca-Activated Channels

2435-Pos Board B405

Impaired Ca^{2+}-Dependent Activation of Large Conductance Ca^{2+}-Activated K^+ Channels in the Coronary Artery Smooth Muscle Cells of Zucker Diabetic Fatty Rats

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The vascular large conductance Ca^{2+}-activated K^+ (BK) channel plays an important role in the regulation of vasoreactivity and vital organ perfusion in response to changes in intracellular metabolic state and Ca^{2+} homeostasis. Vascular BK channel functions are impaired in diabetes mellitus but the underlying molecular mechanisms have not been examined in detail. In this study, we examined and compared the activities and kinetics of BK channels in coronary arterial smooth muscle cells from Lean control and Zucker Diabetic Fatty (ZDF) rats using single channel recording techniques. We found that BK channels in ZDF rats have impaired Ca^{2+} sensitivity, including an increase in the free Ca^{2+} concentration at half-maximal effect on channel activation, reduced steepness of Ca^{2+} dose-dependent curve, altered Ca^{2+}-dependent gating properties with decreased maximal open probability, reduced mean open time, and prolonged mean closed time durations. In the presence of 1 μM free Ca^{2+}, voltage-dependent activation of BK channels was altered in ZDF rats with a 48 mV depolarizing shift in $V_{1/2}$ compared to Lean control. However, the equivalent charge z was not changed and in 0 μM free Ca^{2+}, there was no $V_{1/2}$ shift in ZDF BK channels, suggesting that the impaired voltage-dependent changes were secondary to Ca^{2+}-dependent changes in channel gating properties. In addition, the BK channel β subunit-mediated activation by dehydrosoyasaponin-1 (DHS-1) was lost in cells from ZDF rats. Immunoblotting analysis confirmed that there was a 2.1-fold decrease in BK channel β_1 subunit expression in ZDF rats, compared with that in Lean rats. These abnormalities in BK channel gating lead to increase in the energy barrier for channel activation, and may contribute to the development of vascular dysfunction and complications in type 2 diabetes mellitus.

2436-Pos Board B406

Regulation Of BK Channels By FK506 Binding Protein 12.6 In Vascular Smooth Muscle Cells

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Big-conductance, calcium-activated potassium (BK) channels are important for numerous physiological responses, including relaxation of vascular smooth muscle cells (SMCs). The activity of BK channels can be regulated by several signaling molecules. Here we provide biochemical evidence showing that FK506 binding protein 12.6 (FKBP12.6), an endogenous molecule known to regulate ryanodine receptors/calcium release channels, is physically associated with the BK channel α subunits in mouse cerebral arteries. Inside-out single channels recordings show that application of FK506 to remove FKBP12.6 significantly decreases the open probability of BK channels in freshly isolated mouse cerebral artery SMCs. The effect of FK506 is concentration-dependent. Similar to chemical removal of FKBP12.6 with FK506 exposure, genetic removal of FKBP12.6 with gene deletion produces an inhibitory effect on the activity of single BK channels as well. FKBP12.6 gene deletion also reduces the sensitivity of BK channels to voltage and calcium. Consistent with these results, agonist-evoked vasoconstriction is augmented in isolated arteries from FKBP2.6 gene deletion mice. Moreover, blood pressure is higher in FKBP12.6 gene deletion mice than control mice. In conclusion, our findings for the first time demonstrate that FKBP12.6 is associated with BK channels and regulates the channel functions, which may play an important role in controlling vascular tone and blood pressure.

2437-Pos Board B407

Role of ESCRT Proteins in Controlling the Lysosomal Degradation of KCa3.1 in HEK and Endothelial Cells

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In a previous study we have shown that KCa3.1 is rapidly internalized from the plasma membrane and has a short half-life in HEK293 and endothelial HMEC-1 cells (Biophys. J. 2008 94: 529). The aim of the present work was to investigate the molecular mechanisms controlling this fast degradation of KCa3.1. Using the Biotin-acceptor-KCa3.1 construct, recently engineered in our lab, the channel was fluorescently labeled at the cell surface and the cells were incubated at 37°C for different periods of time. The fate of the endocytosed channels was addressed by confocal microscopy.

After 5 h incubation at 37 °C, almost all protein was degraded, as demonstrated by a very low fluorescence level inside the cells. However, when the same treatment was applied in the presence of lysosomal proteases inhibitors leupeptin/pepstatin, we observed an accumulation of the channel inside the cells, suggesting that lysosomes are involved in KCa3.1 degradation.

Next, we addressed the possible role of the endosomal sorting complex required for transport (ESCRT) components in this process. We have investigated the role of TSG101 (a member of ESCRT-I complex) and SKD1/VPS4 (ESCRT-III). Cells were doubly transfected with Biotin-KCa3.1 and either the wild type construct or a dominant negative form of SKD1/VPS4 (E235Q) and TSG101, respectively. For $SKD1^{E235Q}$ and mutant TSG101 cells, we observed a lack of channel degradation, as compared to control cells.

These results show for the first time the role of ESCRT family proteins in targeting KCa3.1 for lysosomal degradation in HEK and HMEC-1 cells.

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2438-Pos Board B408

Biochemical Evidence of Slo1 Protein Internal Myristoylation: Involvement of a Hydroxyester Chemical Bond

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Myristic and/or palmitic acid incorporation to proteins is a mean by which cells tether proteins to the intracellular leaflet of plasma membranes. Two types of protein myristoylation have been reported; one occurs co-translationally at the N-terminus (e.g. c-Src) and the other post-translationally at an internal amino-acid residue. Here, we tested whether Slo1 might undergo post-translational myristoylation as it lacks an N-terminal consensus site for myristoylation. HEK-293T cells expressing Slo1 or c-Src (positive control) were metabolically radiolabeled with [^{3}H]-myristic acid and subjected to immunoprecipitation; radiolabeled proteins were detected by autoradiography. Our data show that Slo1 incorporates [^{3}H]-myristic acid (n=5) via a post-translational mechanism as assessed by the lack of effect upon inhibition of protein synthesis with cyclohexamide. As control, cyclohexamide treatment reduced c-Src myristoylation confirming its co-translational incorporation (n=3). Next, we sought to determine what type of chemical bond is involved in Slo1 protein myristoylation. Hydroxylamine (NH_2OH) at pH10 but not Tris-HCl at pH10 (negative control) or NH_2OH at pH 7, cleaves hydroxyester bonds. Treatment of **[^{3}H]-**myristoyl-Slo1 with NH_2OH, pH10 but neither treatment with Tris-HCl at pH10 nor NH_2OH at pH7, completely removed incorporated myristic acid from Slo1 (n=3). Possible palmitoylation of Slo1 via a thioester bond was excluded because treatment of labeled Slo1 with NH_2OH at pH7 which cleaves thioester bonds or 1.4 M β-mercaptoethanol, a reducing agent, did not alter the signal. Further, we did not observe Slo1 labeling using [^{3}H]-palmitate (n=2). These data strongly support an involvement of a hydroxyester chemical bond between myristic acid and Slo1 S/T/Y residue(s). In conclusion, we show for the first time that Slo1 protein is post-translationally myristoylated at an internal site. This myristoylation might play a role in controlling Slo1 channel structure, function or trafficking. Supported by NIH.

2439-Pos Board B409

Palmitoylation Controls BK Channel Regulation By Phosphorylation

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Large conductance calcium- and voltage- gated potassium (BK) channels are important regulators of physiological homeostasis and their function is potently modulated by protein kinase A (PKA) phosphorylation. PKA regulates the channel through phosphorylation of residues within the intracellular C-terminus of the pore-forming α-subunits. However, how PKA phosphorylation of the α-subunit effects changes in channel activity are unknown. The STREX variant of BK channels is inhibited by PKA as a result of phosphorylation of a serine residue within the evolutionary conserved STREX insert. As this inhibition is dependent upon phosphorylation of only a single α-subunit in the channel tetramer we hypothesised that phosphorylation results in major conformational rearrangements of the C-terminus. Using a combined imaging, biochemical and electrophysiological strategy we have defined the mechanism of PKA-inhibition of BK channels. We demonstrate that the cytosolic C-terminus of the STREX BK channel uniquely interacts with the plasma membrane via palmitoylation of evolutionary conserved cysteine residues. PKA-phosphorylation of STREX dissociates the C-terminus from the plasma membrane resulting in channel inhibition. Abolition of channel palmitoylation by site-directed mutagenesis or pharmacological inhibition of palmitoyl-transferases prevents PKA-mediated inhibition. Thus PKA inhibition of BK channels is conditional upon the palmitoylation status of the channel. Palmitoylation and phosphorylation are both dynamically regulated thus cross-talk between these two major post-translational signalling cascades provides a novel mechanism for conditional regulation of BK channels. Interplay of these distinct signalling cascades has important implications for the dynamic regulation of BK channels and the control of physiological homeostasis.

2440-Pos Board B410

Bovine and Mouse SLO3 K^+ Channels: Many Functional Differences Map to the Same Region

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Genes pertaining to male reproduction, especially those involved in sperm production, morphologically and functionally evolve much faster than their non-sexual counterparts. *SLO3* is an especially intriguing example of such a rapidly evolving gene. The *SLO3* gene encodes a K^+ channel which is expressed only in mammalian sperm and is evolving much faster than its close paralogue *SLO1* which is expressed in brain and other organs. We cloned the bovine orthologue of *SLO3* (*bSLO3*) and compared its primary sequence and functional properties to its mouse orthologue (*mSLO3*) which we previously cloned. A comparison of bSLO3 and mSLO3 primary sequences showed far less conservation than for SLO1 proteins in mouse and bovine species. Functionally, bSLO3 and mSLO3 also differ markedly with respect to their voltage range of activation, their ion selectivity, and their activation kinetics. Remarkably, although there are many regions of low conservation between bSLO3 and mSLO3 proteins, we found that all of the different functional properties that we measured map to a small region of low conservation in the RCK1 domain. One or more of these different functional properties may reflect differences in the resting membrane potentials of sperm in bovine and mouse species. This work was supported by National Institute of Health grants 1R21HD056444-01A1 to C.M.S. and R24 RR017342-01 and R01 GM067154-01A1 to L.S.

2441-Pos Board B411

Multiple Components of Ca-activated K currents in mouse pancreatic beta cells

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Multiple Components of Ca-activated K currents in mouse pancreatic beta cells
In beta cells, two types of Ca activated K channels have been described. One component, K_{slow}, is believed to be mediated by small-conductance, voltage-independent (SK) channels; it is thought to regulate the duration of intervals between successive action-potential bursts observed when beta cells are exposed to moderately elevated glucose. In contrast, the functional role of the second type of Ca activated K channels, the large conductance, Ca- and voltage-activated, BK channels, is not well understood. BK channel subunit genes have been detected in insulin-secreting cell lines, and BK channels have been observed functionally in rodent beta cells; however, early studies with BK channel blocking drugs have failed to identify a role for these channels in the electrical excitability or the stimulus-secretion coupling of beta cells.
Using patch clamp recording under quasi-physiological conditions, we show that the BK channel current can contribute up to a half of the outward current activated by depolarizing pulses whose amplitude resembles the voltage excursion of beta cell action potentials. Kinetic and pharmacological experiments reveal that the beta cell BK current consists of several pharmacologically, kinetically, and possibly spatially, distinct components. Our results suggest that the BK current could play a significant role in regulating beta cell electrical excitability of stimulus secretion coupling.

2442-Pos Board B412

Calcium Binding Causes A Conformational Change in The RCK1 Domain of The BK(Ca) Channel

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Calcium plays a major role in controlling the opening and closing of the large conductance BK_{Ca} channels. Two high affinity binding sites have been identified in the channel structure and one of these sites is the DRDD loop in the N-terminus of the RCK1 domain. Mutation of the first aspartate in this conserved DRDD motif significantly reduces Ca^{2+} sensitivity and hence this residue has been implicated as a coordinating group in the binding site. Here we present results on the prediction of the Ca^{2+} binding site based on a series of detailed computational studies. The basic protocol involves multiple iterations of random ion placement, implicit solvent molecular dynamics simulations and statistical analysis. Our resulting model matches very well with existing mutagenesis data, and subsequent explicit solvent molecular dynamics simulations have been performed using this Ca^{2+} bound structure. Comparison of the dynamics and conformations of the Ca^{2+} bound and unbound simulations reveal a concerted conformational change in the structure and suggest a potential mechanism for calcium dependent activation of these channels.

2443-Pos Board B413

Comparative Mechanisms Of Activation Of The Slo1 BK Channel By Ca2+ And H+ Mediated By The RCK1 Domain

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Large-conductance Ca2 ± and voltage-gated K+ (Slo1 BK) channels are allosterically activated by depolarization and intracellular Ca2+. High-affinity activation of the channel by Ca2+ involves two sites, the Ca2+ bowl sensor and the RCK1 sensor, the latter of which is also required for the stimulatory action of intracellular H+ (Hou et al., Nat Struct Mol Biol. 15, 403, 2008). We investigated the comparative effects of Ca2+ and H+ on activation of the Slo1 BK channel mediated by the RCK1 sensor using a Ca2+ bowl-defective mutant expressed in HEK cells. Decreasing pHi from 7.5 to 6.2 shifted the voltage-conductance (GV) curve to the left by ~50 mV. The shift in GV by H+ was, however, only ~40% of that caused by a saturating concentration of Ca2+ in the mutant. Single-channel measurements at negative voltages where voltage sensor activation is negligible verified that 200 uM Ca2+ drastically increased open probability, corresponding to the allosteric coupling factor C = ~4 in