lead optimization programs at Exelixis. Whole-cell recordings from an in-house developed cryopreserved CHO cell line heterologously expressing HERG channels were obtained with IonWorksQuattro™. Ion channel expression was unaltered for >10 months (>400 pA/well). Only population patch-clamp wells with seal test >25 MΩ and peak tail currents >100 pA were selected for analysis. Success rates consistently obtained were >90%. Pharmacological analysis, characterized by automated IC50 determinations, were compared with equivalent studies performed with a Giga-Ω seal manual patch-clamp system to evaluate the predictive competency of the automated instrumentation and then validate its effective impact on quantitative SAR analysis for the selection and prioritization of lead compounds, avoiding potential QT prolongation liabilities at early stages.

1652-Pos
Electrostatic Tuning of Cellular Excitability
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Voltage-gated ion channels regulate the electric activity of excitable tissues like the heart and the brain. Therefore, treatment for conditions of disturbed excitability is often based on drugs that target ion channels. Traditional ion-channel drugs aim at plugging the ion-conducting pore. We instead propose a novel pharmacological mechanism for how to regulate channel activity by targeting the voltage sensor of voltage-gated K channels. By studying the effect of different free fatty acids and fatty acid derivatives we show that charged lipophilic substances can tune channel opening and consequently excitability by an electrostatic interaction with the channel. Polyunsaturated fatty acids shift the voltage dependence of activation of the Shaker K channel in hyperpolarizing direction. The negative carboxyl charge is crucial for the effect. A positively charged arachidonic acid derivative (arachidonyl amine) was synthesized and shown to instead shift the voltage dependence in depolarizing direction. Thus, the direction of the effect on the channel's voltage dependence is determined by the charge of the substance. Uncharged methyl esters of polyunsaturated fatty acids do not affect the voltage dependence. Computer simulations of membrane excitability demonstrate that small changes in the voltage dependence of Na and K channels have prominent impact on excitability and the tendency for repetitive firing. For instance, a shift in the voltage dependence of a K channel with −5 or +5 mV corresponds to a three-fold increase or decrease in K channel density, respectively. We suggest that electrostatic tuning of ion channel activity can be a new and powerful pharmacological approach to affect cellular excitability.

1653-Pos
Arming Antibodies for Subtype-Selective Photo-Inhibition of Voltage Gated Potassium Channels
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Establishing the molecular identity of native voltage-gated potassium (Kv) channels has been a particularly challenging problem. Mammalian Kv channels arise from a family of more than 40 genes and few inhibitors are selective for any one Kv subunit type. The identification of channel types underlying native ionic currents has been greatly aided by the availability of subtype specific inhibitors, but drugs of great selectivity have not yet been discovered for most Kv subunits. There exist, however, extensively characterized monoclonal antibodies against extracellular S1-S2 linker epitopes that exhibit clear specificity for Kv4.2, Kv2.1 or Kv1.1. Unfortunately, none of these antibodies have been found to inhibit channel currents.
A proven strategy for targeted inhibition of proteins is to label antibodies with chromophore ''warheads'' that induce oxidative damage to the target protein upon illumination. Photo-stimulation of certain chromophores leads to the generation of singlet oxygen, which has a 40 Å half-maximal radius of oxidative damage, suitable to oxidize the protein target when conjugated to an antibody. Porphyrins are amongst the most efficient photo-induced generators of singlet oxygen known, having greater extinction coefficients, lower sensitivity to photobleaching, and higher quantum yields for singlet oxygen than compounds, such as fluorescein, classically used for targeted photo-inhibition of proteins. We have synthesized a series of porphyrin derivatives that irreversibly inhibit Kv4.2 or Kv2.1 currents upon illumination. Covalent attachment of porphyrin to an anti-Kv4.2 antibody has resulted in selective inhibition of Kv4.2 at a 10 nM concentration. By attaching warheads to subtype-selective antibodies, we aim to find a serial solution to the problematic dearth of subunit-specific Kv inhibitors.

Anion Channels

1654-Pos
Cholesterol Depletion Facilitates Recovery from Hypotonic Cell Swelling of CHO
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The maintenance of cell volume homeostasis prevents pathological cell swelling that can lead to severe cellular dysfunction or death. A key step in maintaining cell volume in many cell types is activation of volume-regulated anion channels (VRAC). Our earlier studies showed that activity of VRAC is facilitated by a decrease in cellular cholesterol (Levitan et al 2000). These observations suggest that lowered cholesterol should also facilitate regulatory volume decrease (RVD), a process used by cells to recover from hypotonic swelling. The main constraint in testing this prediction, however, has been the lack of adequate methods to rapidly and reproducibly measure changes in cell volume of substrate-attached cells. In this study, we address this question using a novel microfluidic methodology from Reichert Inc. (CVC-7000), to measure cell volume response to hypotonic challenges (30% osmotic gradient) in real time. Cholesterol depletion facilitated the recovery from swelling via a more rapid onset of RVD (~130 s vs. 215 s in control and cholesterol depleted cells, respectively) and a higher degree of volume recovery after 10 min (41% ± 6% vs. 65% ± 6% in control and cholesterol depleted cells, respectively). In contrast, enriching the cells with cholesterol had no effect on RVD. These observations are consistent with our previous studies showing that while cholesterol depletion increases cell stiffness, cholesterol enrichment has no effect (Byfield et al 2004). These observations suggest that cholesterol depletion, and the consequent increase in cell stiffness, facilitates RVD by enhancing the activity of VRAC.

1655-Pos
Expression and Novel Function of Bestrophin-2 in Goblet Cells in Mammalian Colon
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Anion channels and transporters in the gastrointestinal epithelium play essential roles in fluid secretion and absorption and participate in regulating the pH and ionic composition of the gut luminal contents. Diarrheas produced by bacterial enterotoxins such as cholera and rotavirus are associated, respectively, with activation of two kinds of Cl- channels, the cystic fibrosis transmembrane conductance regulator (CFTR) and Ca2+-activated Cl- channels (CaCCs). Although the roles and mechanisms of CFTR are relatively well understood, CaCCs have remained enigmatic partly because their molecular identity has remained in question. Here we have investigated the role of bestrophin-2, a candidate CaCC protein, in colon using a mouse knockout model. Best2-/- knockout mice exhibit a greatly reduced amplitude of cholinergic (Ca2+)-stimulated anion secretion, consistent with Best2's potential role as a CaCC. However, unexpectedly, Best2 is expressed in the basolateral membrane of mucin-secreting colonic goblet cells and not in the apical membrane of colonocytes as predicted if it was a CaCC. Analysis of the cholinergically-stimulated anion secretion revealed that a large fraction of the current was carried by HCO3-, was unaffected by CFTR blockers, and was carried by Best2 channels. Whole cell patch clamp analysis of isolated colonocytes revealed two kinds of Ca2+-activated Cl- channels, currents with linear I-V relationships carried by Best2 and reduced in the knockout and outwardly-rectifying currents that resemble currents carried by TMEM16A, another candidate CaCC protein that is expressed on the apical membrane of surface colonocytes and is probably involved in Cl- absorption. These results provide a new perspective on cholinergic regulation of colonic secretion and may have relevance to colitis and inflammatory bowel disease, two diseases that exhibit defective anion transport. Further, they provide new insights into the functions of the enigmatic bestrophin family of anion channels.

1656-Pos
TMEM16A is Expressed in Vascular Tissues that Display Robust Calcium-Activated Chloride Currents
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Calcium activated chloride (Cl_{Ca}) channels are an important contractile mechanism in smooth muscle cells. Activation of these channels by calcium (Ca^{2+}) ions leads to Cl^- efflux and membrane depolarization. This depolarization then favors the activation of voltage-gated Ca^{2+} channels (e.g. L-type), providing a positive feed-back loop that allows for sustained contraction.

The molecular candidate encoding for this channel has remained elusive, although a number of suggestions have been investigated. The latest contender to emerge is TMEM16A (Anoctamin). Evidence so far confirms that over-expression of TMEM16A generates Cl^- currents with the same time-dependent kinetics and Ca^{2+} sensitivity as recorded through Cl_{Ca} channels in smooth muscle cells.
This study investigated expression profiles of TMEM16A in a number of vascular tissues across a number of species. The aim of this study was to confirm the role of TMEM16A as a potential candidate encoding for Cl_{Ca} currents (I_{ClCa}) in native vascular myocytes.
Smooth muscle cells were enzymatically isolated from the mouse portal vein (PV), aorta and carotid artery (CA), rat pulmonary artery (PA), and the rabbit PA. Robust I_{ClCa}, exhibiting distinctive voltage-dependent kinetics, were recorded from all of the tissue preparations mentioned.
RT-PCR was performed, using species-specific primers designed against the available sequences for TMEM16A. Mouse aorta, PA, CA and PV, rat aorta, PA and PV, and rabbit PA tissues were studies. All samples showed clear expression of TMEM16A.
Immunocytochemistry revealed specific expression of TMEM16A in rat PA, and mouse PV, CA and aorta isolated myocytes. This was apparent throughout the cytoplasm, with punctuate ''hotspots''. Western blot analysis showed bands of the expected molecular weight in mouse aorta, PV and CA.
In summary, we have demonstrated that TMEM16A is a viable candidate for the encoding of Cl_{Ca} channels in vascular smooth muscle.

1657-Pos
TMEM16A is a Calcium-Activated Chloride Channel in Pulmonary Artery Smooth Muscle Cells
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Calcium-activated chloride channels (CaCCs) play pivotal roles in many physiological processes. In vascular smooth muscle, activation of these ion channels by agonist-induced Ca^{2+} release provokes membrane depolarisation, increased Ca^{2+} entry through L-type Ca^{2+} channels and ultimately vasoconstriction. The molecular identity of vascular CaCCs is not fully understood. Here we present evidence that TMEM16A (or Anoctamin1), a member of the transmembrane 16 (TMEM16) protein family forms CaCCs in pulmonary artery smooth muscle cells (PASMCs). Patch-clamp analysis in acutely isolated PASMCs revealed strongly outward rectifying Ca^{2+}-activated Cl^- currents which activated slowly at positive potentials and showed large deactivating tail currents upon repolarisation, very similar to heterologous TMEM16A currents (Caputo *et al*. (2008) *Science* 322, 590-594; Yang *et al.* (2008) *Nature* 455, 1210-1215; Schroeder *et al.* (2008) *Cell* 134, 1019-1029). High levels of TMEM16A mRNA were identified in rat pulmonary arteries and various other vascular smooth muscle cell types. Downregulation of TMEM16A gene expression in primary cultured PASMCs, with small interfering RNA, was accompanied by almost total loss of whole-cell CaCC currents. Our data suggest that TMEM16A forms calcium-activated chloride channels in rat pulmonary artery smooth muscle.

1658-Pos
Regulation and Gating of mAno1 by Voltage and Calcium
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Ca-activated chloride channels play important roles in epithelial secretion, regulation of vascular tone, control of membrane excitability, olfactory transduction, and photoreception. Recently, Ano1 (TMEM16a) has been identified as a Ca2+-activated chloride channel. Activation of Ano1 exhibits both Ca2+- and voltage- dependence. However, the structures and mechanisms responsible for Ca2+- and voltage-dependent activation remain unknown. mAno1 exhibits a strong outward rectification at <0.5 μM Ca2+ concentration with little inward current. As Ca2+ concentration is increased, inward current increases. We hypothesized that Ano1 has two Ca2+ binding sites, a high affinity site that controls the outward current and a low affinity site that controls inward current. We further hypothesized that a region in the first intracellular loop characterized by five contiguous glutamates (444EEEEE448) was the potential high-affinity Ca2+ sensor. To test this, HEK-293 cells were transfected with wild type mAno1 and mutants in the region surrounding the five glutamates and studied by whole cell and inside-out excised patch recording. In the absence of intracellular Ca2+, wild type mAno1 could be activated by very strong depolarizations ($> +100$ mV). In the presence of Ca2+, the G-V relations were well fit with the Boltzmann equation. V1/2 was 73 mV at 10 μM intracellular free Ca2+. Increasing Ca2+ shifted the G-V relation to the left. We mutated the putative Ca2+ binding site by deleting the last glutamate of the cluster plus the 3 trailing amino acids (del448EAVK451), substituting the first four glutamates with alanines (444EEEE/AAAA447), and substituting of each negatively charged amino acid with alanine. These mutations altered both voltage-dependent and Ca2+-dependent gating of the channel in complex ways that are consistent with this region being an integral component of Ca2+ dependent gating of the channel.

1659-Pos

WITHDRAWN.

1660-Pos
The Preference of the CLC-0 Pore for Charged Methanethiosulfonate Reagents
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Previous experiments from our laboratory showed that the negatively-charged methanethiosulfonate (MTS) reagent, 2-sulfonatoethyl MTS (MTSES) modified a cysteine residue at the Y512 position of CLC-0 faster than the positively-charged 2-(trimethylammonium)ethyl MTS (MTSET). This observation suggested a hypothesis that the pore of CLC-0 may be built with a positive intrinsic pore potential. The hypothesis, however, is challenged by our most recent finding that the preference for the negatively charged MTS reagent is significantly reduced when the cysteine is placed at a deeper pore position, E166. In this study, we examine the discrepancy in the preference for charged MTS reagents between the Y512C and E166C mutants. We find that the inhibition of E166C by intracellularly-applied MTS reagents is contaminated by the modification of an endogenous cysteine because MTS modification rates of the E166A and E166C mutants are similar to each other. We identify that C229, which is located at the dimer interface of the channel, is the endogenous cysteine responsible for this contamination. After C229 is mutated, CLC-0 resumes a preference for selecting the negatively-charged over the positively-charged MTS reagents in modifying E166C, re-confirming the idea of a positive intrinsic potential in the pore. Our study also suggests a communication between the pore region near E166 and the dimer interface near C229 because the inhibition of the channel due to the modification of C229 is dependent upon the amino acid placed at position 166.

1661-Pos
Regulation of the Chloride/Proton Exchanger CLC-5 By Internal pH
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ClC-5 transports anions and protons across intracellular membranes and is necessary for endosomal/lysosomal acification. Similar to other ClC transporters, ClC-5 can switch between two different transport modes and operate either as anion-proton exchanger or as anion channel, depending on the type of external anion. So far, only some regulatory mechanisms that affect the functional mode of these transporters have been described. We here use combined whole-cell patch clamp and fluorescent intracellular pH recordings in transfected tsA201 cells to characterize ClC-5 mediated transport under various ionic conditions. Asymmetric lowering of intracellular pH results in small increases of both total and proton current amplitudes that might be well explained by changes in the electrochemical gradient imposed by such a maneuver. With internal Cl^--based solution (pH 7.4) exchange of external Cl^- with SCN^- results in ~5-fold increase in total ClC-5 currents at positive voltages. At higher internal proton concentration (pH 6) the same change of external anion composition results in a 70-fold increase in total currents. The voltage dependence of channel opening is not significantly altered, excluding shifts of the activation curve as a mechanistic explanation. For mutant ClC-5 lacking the ''proton glutamate'', E268H ClC-5, the simultaneous effect of internal protons and external SCN is significantly less pronounced. These findings imply that acidic internal pHs change the protonation state of the proton glutamate and promote the action of external uncoupling anions. In addition, they imply that ClC-5 might even mediate uncoupled anion transport at physiological anion compositions. ClC exchangers may utilize part of the proton electrochemical driving force between the intra/extravesicular sites of intracellular compartments to establish anion concentration gradients. The described effects of intracellular pH might be an important part in this pathway and contribute to regulation of endosomal/lysosomal physiological function.